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PRINCIPAL INVESTIGATOR: William J. Muller, M.D.

CONTRACTING ORGANIZATION: McMaster University
Hamilton, Ontario, Canada L8S 4K1

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6. AUTHOR(S)
William J. Muller, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
McMaster University
Hamilton, Ontario, Canada L8S 4K1

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Transgenic mice expressing an activated neu oncogene in the mammary epithelium develop metastatic mammary tumors within a short latency period. Given our recent observations suggesting that co-activation of the epidermal growth factor receptor and Neu in the mammary epithelium of transgenic mice results in the synergistic transformation of the mammary epithelium, we decided to assess whether a functional EGFR was required for Neu-induced tumors. To accomplish this objective, we have recently interbred these activated Neu strains to a naturally occurring mouse mutation possessing a catalytic defective EGFR (Waved-2). The preliminary results of these analyses revealed that while a functionally active EGFR was not absolutely required for Neu induced tumorigenesis, inactivation of its catalytic activity appears to affect tumor growth rate. The observed cooperativity of EGFR and Neu may in part be due the ability of the EGFR and Neu to recruit distinct but complementary signaling pathways. In fact Neu can recruit the Src signaling pathway to EGFR/Neu complexes through a direct and specific binding to the catalytic domain of Neu. We are currently exploring the role of other EGFR family members in Neu mediated mammary tumors by crossing the MMTV/neu mice to the recently established MMTV/erbB-4 mice.

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INTRODUCTION

The Epidermal Growth Factor Receptor (EGFR) family comprises four closely related type 1 receptor tyrosine kinases (RTK's) (EGFR, Neu [erbB-2,HER2], erbB3[HER3], and erbB-4[HER4]) that are receptors for a variety of mitogenic growth factors (Ullrich and Schlessinger, 1990). Enhanced expression of the various EGFR family members has been implicated in the genesis of human breast cancer. For example, amplification and consequent over expression Neu has been observed in a significant proportion of human breast cancer (King et al., 1985, Van de Vjier et al., 1987, Slamon et al., 1987, Slamon et al., 1989). Moreover, the extent of Neu over expression has been also inversely correlated with patient survival (Paterson et al., 1991, Gullick et al., 1991). More recent studies have implicated other EGFR family members including EGFR, HER3 and HER4 in the genesis of human breast cancer (Lacroix et al., 1988, Kraus et al., 1989 Plowman et al., 1990, Plowman et al., 1993).

In addition to the detection of elevated expression of these EGFR family members, the activity of these receptors can be influenced by the expression of specific polypeptide ligands for these various EGFR family members. For example, the expression EGFR specific ligands Transforming growth factor α (TGFα) and Epidermal growth factor (EGF) can be detected in primary human breast cancers (Salomon et al., 1990). Moreover, the expression some families of erbB-3 and erbB-4 ligands known collectively as the NDFs (Neu differentiation factors [NDFs]) have also been implicated in the pathogenesis of breast cancer (Holmes et al., 1992, Wen et al., 1992). Although Neu cannot directly interact with either the NDFs or EGF ligands, its tyrosine kinase activity can be profoundly influenced by expression of these growth factors. For example, Neu is a substrate of the activated EGFR following stimulation of cells with EGF or TGFα (Stern and Kamps, 1988, Kokai et al., 1988, Goldman et al., 1990) Similarly, Neu can be transphosphorylated by either erbB-3 or erbB-4 following stimulation of mammary tumor cells with the NDFs (Karunagraran et al., 1996). The ability of these growth factors to modulate the activity of Neu is thought to mediate through the formation of specific heterodimers of Neu and the different EGFR family members (Wada et al., 1990). Consistent with these observations, co-expression of Neu and EGFR or Neu and erbB3 result in the efficient transformation of fibroblasts in vitro (Kokai et al., 1989).

More recently, it has been demonstrated that retention of Neu in the endoplasmic reticulum, through the expression of Neu specific single chain antibody, can interfere with ability of breast cancer cells to respond to the mitogenic stimulation of both EGF and NDFs (Graus-Porta et al., 1995). The central importance of HER2 in signaling by EGF or NDF is further highlighted by the observation that heterodimers between either EGFR and Neu or erbB3/erbB4 and Neu results in the induction of high affinity receptors for these potent growth factors (Karunagaran et al., 1996, Wada et al., 1990). Taken together, these observations suggest the interaction of these different EGFR family members play a critical role in the induction of mammary tumors.

Direct evidence for the importance of these EGFR family members and their cognate ligands

in the induction of mammary tumors derives from studies of transgenic mice that have been engineered to over express these genes in the mammary epithelium. For example, elevated expression of a constitutively active form of Neu resulted in the rapid induction of multi focal mammary tumors which appeared within a short latency period (Muller et al., 1988). The mammary epithelial specific expression of a wild-type Neu also resulted in the induction of mammary tumors in transgenic mice (Guy et al., 1992). However, in contrast to rapid development of mammary tumors observed in mice expressing the constitutively active form, the tumors arising in the wild-type strains were focal in origin and appeared after a long latency period (Guy et al., 1992). Significantly, the induction of mammary tumors in the wild-type neu mice correlated in many of the tumor samples with the occurrence of activating mutations in the transgene (Siegel et al., 1994). These observations suggest that the catalytic activation of Neu is critical in the induction of a mammary carcinoma.

Additional evidence supporting a role of EGFR family in mammary tumorigenesis derives from observations with transgenic mice expressing several EGFR family ligands. Transgenic mice expressing TGFα in the mammary epithelium develop global mammary epithelial hyperplasias which occasionally progress to overt mammary carcinomas (Matsui et al., 1989, Jhappan et al., 1989, Sandgren et al., 1989). More recently, mammary epithelial expression of NDF has been reported to result in the induction of mammary tumors (Krane and Leder, 1996). However like the wild-type neu transgenic strains, the induction of mammary tumors in these strains appears to require additional genetic events. The activity of the EGFR receptor family has also been implicated as an important factor in normal mammary epithelial development. For example, a naturally occurring mutation the EGFR receptor which results in a severe impairment of EGFR kinase activity leads to a dramatic lactation defect in Waved-2 mice (Lutteke et al., 1994, Fowler et al., 1995).

The primary purpose of our Army-sponsored research program has been to elucidate the role of various EGFR family members in the induction of mammary cancers in vivo. Our initial objective was to assess the role of activation of the EGFR in Neu induced mammary tumorigenesis. Given the observation that Neu and the EGFR and its cognate ligands are frequently co-expressed in primary breast cancers, we have crossed separate strains of transgenic mice carrying either a MMTV/wild-type neu or MMTV/TGFα fusion gene to generate dual carriers that co-express both neu and TGFα in the mammary epithelium. The results of this study which have recently been published in Molecular Cellular Biology (Muller et al., 1996, see appended manuscript #1) demonstrated that bigenic animals that co-expressed TGFα and Neu developed multi focal mammary tumors within a significantly shorter latency period than either parental strain alone. The induction of tumors in these bigenic strains was further correlated to tyrosine phosphorylation of Neu. However unlike the parental wild-type neu strains, the catalytic activation of Neu occurred in the absence of neu mutations (Muller et al., 1996.). We were further able to demonstrate that the induction of tumors was also correlated with ability of Neu to recruit the c-Src family kinase family (Muller et al., 1996). In a separate series of studies, we showed that the ability of c-Src to couple with EGFR/Neu heterodimer was due the specific interaction of c-Src to directly couple with Neu

following EGF stimulation (Muthuswamy and Muller, 1995a, see appended manuscript #2). Moreover, we were also able to demonstrate that activation of the c-Src pathway in Neu induced tumors correlated with their association with distinct sets of tyrosine phosphorylated proteins in vivo (Muller and Muthuswamy, 1995b, see appended manuscript #3).

One of our research objectives in the second year of funding from the Army has focused on assessing whether a catalytically inactive EGFR present in the Waved-2 background (Lutteke et al., 1994) is required for neu-induced mammary tumorigenesis. To accomplish this objective, we have crossed the Waved-2 mice to transgenic mice expressing a constitutively active form of neu in the mammary epithelium. Although the results of these analyses are ongoing, preliminary observations suggest that a catalytic active EGFR is not absolutely required for the induction of mammary tumors (see Results section). However, these preliminary analyses suggest that the Neu induced tumors to grow more slowly in the Waved-2 genetic background.

Another important research goal in the second year of funding was to identify the region within Neu that is responsible for the differential binding of Src to Neu rather than the EGFR. To this end we have tested the ability of chimeric receptors containing various components of the Neu and EGFR catalytic domains. The results of these analyses revealed that the Src family kinases appear to bind within a carboxyl portion of the Neu catalytic domain (see Results section). Finally in the last year we have generated transgenic mice that express erbB-4 in the mammary epithelium. Although these mice have yet to develop mammary tumors, we are currently breeding these mice to the MMTV/wild-type neu mice to assess whether co-expression of Neu and erbB-4 can result in the acceleration of tumor formation. In addition we are also assessing whether co-expression of erbB-4 and its cognate ligand NDF can result in the acceleration of mammary tumors. To accomplish we have initiated a collaboration with Dr. Phil Leder to assess the consequence of interbreeding the MMTV/erbB-4 mice with transgenic mice expressing the MMTV/NDF strains (Krane and Leder, 1996).

RESULTS:

A catalytically inactive EGFR is dispensable for Neu mediated tumorigenesis.

In our previous year we demonstrated that co-expression of the EGFR ligand, TGFα and Neu in mammary epithelium, resulted in the rapid induction of multi focal mammary tumors (Muller et al., 1996, see appended manuscript). Biochemical analyses of these tumors further revealed that observed cooperativity between Neu and EGFR likely involves receptor transactivation as the mechanism (Muller et al., 1996). Given that Neu and EGFR can interact in a synergistic fashion to transform the mammary epithelium, we decided to test whether the intrinsic kinase activity of the EGFR was required for the rapid induction of mammary tumors. To accomplish this objective we have taken advantage of a natural occurring mouse mutation known as Waved-2 in which EGFR possesses a mutation in the catalytic domain rendering it functionally inactive (Lutteke

et al., 1994). Although the Waved-2 mutation does affect viability, female Waved-2 mice exhibit a lactation defect suggesting that a functional EGFR is required for normal mammary epithelial development (Fowler et al., 1995).

As a first step in assessing the relative importance of EGFR in Neu mediated tumorigenesis, it was necessary to derive a transgenic mouse Neu model which developed mammary tumors in a predictable and synchronous manner. Because the original MMTV wild-type neu mice developed tumors in a stochastic fashion and with variable onset (Figure 1, N#202 strain), this model was not ideally suited to cross to the Waved-2 background since meaningful interpretation of the cross would require the generation of large numbers of animals. Furthermore, molecular analyses of mammary tumorigenesis in the MMTV/wild-type neu mice indicated that the source of this variability in onset of tumors in the wild-type neu mice was likely the requirement for the occurrence of activating somatic mutations in the transgene (Siegel et al., 1994). To address this concern we decided to derive transgenic mice which express one these activated neu alleles under transcriptional control of the MMTV promoter (Figure 2A). This activated neu allele contains a 12 amino acid in-frame deletion in the juxtatransmembrane of Neu resulting in its oncogenic activation. As expected, RNase protection analyses revealed that the transgene was expressed primarily in the mammary epithelium of female and the male reproductive tract of the males (Figure 2B). In contrast to the highly variable onset of mammary tumor formation observed in the MMTV/wild type neu mice (Figure 1, NDL strain), mammary epithelial specific expression of this activated neu allele resulted in the induction of multi focal mammary tumors which occurred in a synchronous fashion (Figure 1). For nomenclature purposes, we refer to these MMTV/activated neu strains as the NDL (Neu deletion) strain The induction of mammary tumors in these strains was correlated with high levels of expression of the activated neu allele (Figure 2B). Moreover Immunoprecipitation/immunoblot analyses of protein lysates with Neu specific antisera and anti-phosphotyrosine specific antibodies revealed that these tumors possessed elevated levels of tyrosine phosphorylated Neu (Figure 3).

Once having established NDL tumor model, we have crossed it to the naturally occurring Waved-2 mutation. To date we have generated 15 female animals that carry the NDL transgene in a Waved-2 homozygous background and 35 other females that carry the NDL transgene in either a Waved-2 heterozygous or wild type background. Although we are still early in the analyses, 2 NDL/homozygous Waved-2 mice and 2 NDL/heterozygous Waved-2 have developed multifocal mammary tumors at approximately 200 days of age. Although the both sets of genotypes developed palpable tumors at approximately the same age, one striking difference between the NDL/waved-2 homozygous animals and NDL/heterozygous animals is that there was a marked difference in tumor growth between the two genotypes. As shown in Figure 4A, the mammary tumors in an age matched sibling carrying the NDL transgene in Waved-2 homozygous background grew very slowly whereas its littermate carrying the NDL transgene in a Waved-2 heterozygous background grew quite rapidly. To confirm that this difference in tumor growth between these strains was not due to differences in transgene expression, we performed RNase protection analyses on 10 ug of total RNA derived from tumors obtained from either genotype with a transgene-specific probe. The results showed that

tumors derived from either NDL/heterozygous or NDL/homozygous genotypes expressed equivalent levels of NDL transcript (Figure 4B). Although these data are preliminary in nature, these observations suggest that while EGFR function is dispensable for the induction of mammary tumors by Neu, its activity may influence tumor growth. Further analyses of the NDL/Waved-2 cross should provide important insight into the role of the EGFR in Neu induced tumorigenesis (see Conclusions and Future Directions).

The differential interaction of the Src family kinases with the EGFR and Neu resides in the catalytic domain of Neu.

In our previous renewal report, we presented evidence that activation of the Src family kinases by either the activated EGFR or Neu involved the direct and specific interaction of the Src family kinases with Neu (Muthuswamy and Muller, 1995, see appended manuscript#2). Given that the interaction of c-Src and Neu occurs through the interaction of c-Src Src Homology 2 (SH2) domain and a phosphotyrosine residue located on Neu (Muthuswamy and Muller, 1995b), we initially examined whether a mutant Neu molecule lacking the 5 known tyrosine phosphorylation sites was capable of interacting with c-Src. This mutant known as NT-NYPD contains phenylalanine substations at each of the known tyrosine phosphorylation sites in the context of activating point mutation in the transmembrane domain (Figure 5C). Analyses of Neu immunoprecipitates from this mutant using a radiolabeled glutathionine-s-transferase c-Src SH2 fusion protein revealed that the NT-NYPD mutant (or mutants harboring single tyrosine phosphorylation sites, NT-YA to NT YE) still retained the capacity to bind the radiolabeled SH2 probe (Figure 6A, lanes 8-13). Consistent with these far western analyses, immunoprecipitation/immunoblot analyses with Src and Neu specific antisera revealed that the NT-NYPD mutant was still able to associate with c-Src (Figure 6C, lane 3). Taken together these observations suggested that the major Neu tyrosine autophosphorylation sites are dispensable for the c-Src binding.

The data generated thus far suggest that c-Src is binding to region distinct from the carboxyl domain which harbors the major tyrosine autophosphorylation sites. To further localize the region in Neu responsible for differential interaction of the Src family kinases, chimeric receptors comprising different segments of the EGFR and Neu were obtained from the laboratory of Dr. P. DiFiore (DiFiore et al., 1990). As shown in Figure 7, all chimeric receptors possess the EGFR ligand binding domain and carboxyl region harboring the EGFR major tyrosine autophosphorylation sites. However, the various chimeric receptors possess different segments of the Neu/erbB-2 catalytic domain and transmembrane domain (Figure 7). In order to assess whether the catalytic domain of Neu was responsible for mediating the interaction of Neu with the Src family kinases, stable Rat-1 cell lines expressing these chimeric receptors were derived. To confirm that these cell lines were expressing selected cell lines expressing the different chimeric receptors were subjected to immunoblot analyses with EGFR specific antisera (Figure 8). In addition to cells expressing the wild type EGFR (WT, RHER) stable cell lines expressing comparable levels of the chimeric receptor

(TMTK, TK, TK1, RT) were selected for further analyses.

Because the chimeric receptors harbor the EGFR ligand domain their activity can be stimulated by addition of EGF ligand. As shown in Figure 9B, addition of EGF to these various cell lines results in a marked stimulation of tyrosine phosphorylation of EGF. Interestingly two of the cells line expressing chimeric receptors possessed high levels of basal tyrosine phosphorylated EGFR even in the absence of EGF stimulation (TMTK, TK, Figure 9B, lanes 2 to 6). To assess whether these different chimeric receptors associated with c-Src, protein lysates from these cell lines was subjected to immunoprecipitation/immunoblot analyses with Src and EGFR specific antisera. The results of these analyses revealed that both the TMTK and TK chimeric receptors associated with c-Src (Figure 9A, lanes 3-6). Because of the elevated basal level of tyrosine phosphorylated EGFR in the unstimulated TMTK and TK expressing cells, association of c-Src with these chimeric receptors was detected under both stimulated and unstimulated conditions. In contrast to these observations stable-Src complexes with the TK1, RT chimeric receptors or wild type EGFR receptors (RHER, EGFR) could not be detected (Figure 9A). Taken together these observations suggest that the c-Src binding site in Neu resides in the Neu catalytic domain. Moreover, since the TK1 chimeric receptor which lacks the carboxyl segment of the Neu catalytic domain failed to bind c-Src, these observations argue that the Src binding site must require these Neu sequences. However the precise identification of the Src binding site on Neu awaits further molecular analyses (see Conclusions and Future Directions).

Derivation and characterization of transgenic mice expressing the MMTV/erbB-4 fusion gene.

Another major focus of our efforts during the last year has been to derive transgenic mice expressing other members of the EGFR family in the mammary epithelium. Biochemical analyses of tumors derived from the MMTV/neu mice revealed that these tumors express high levels of erbB-4 (Muthuswamy and Muller, 1995, see appended manuscript #2). These observations suggest that elevated expression of erbB-4 may contribute to Neu induced tumorigenesis. To directly assess the role of erbB-4 in mammary tumorigenesis, we decided to derive transgenic mice that express elevated levels of erbB-4 in the mammary epithelium. To accomplish this we inserted a cDNA encoding the murine erbB-4 under the transcriptional control of the Mouse Mammary Tumor Virus LTR (Figure 10A) and microinjected this fusion gene into one cell mouse zygotes. Using this approach, a total of eight MMTV/erbB-4 founder animals have been generated. Of the eight founder animals, 7 passed the transgene in the Mendelian fashion. The remaining founder animal failed to pass the transgene.

To further investigate which of these MMTV/erbB-4 transgenic founder animals expressed the MMTV/erbB-4 transgene in a mammary epithelium. To this end 10 ug of total RNA from mammary glands from virgin mammary glands was subjected to RNase protection analyses with a riboprobe specific to the 3' SV40 polyadenylation splicing sequences present in the transgene. The results of these analyses revealed that of the seven transgenic lines examined in these RNase

protection analyses, two exhibited evidence of expression of the transgene in the mammary epithelium with the erbB-4-6 strain expressing the highest in both virgin and multiparous mammary tissue (Figure 10B, lanes 8-9). Interestingly, high levels of expression of erbB-4 transgene were also noted in the testes of four of the male transgene carriers. These data argue that at least two of the MMTV/erbB-4 strains that have been derived are expressing the MMTV/erbB-4 transgene in the mammary epithelium. We are currently monitoring females from these four lines for the appearance of mammary tumors. Although we are still early in the analyses, the oldest females (approximately six months of age) have yet to display evidence of tumors. To ascertain whether the erbB-2 and erbB-4 signaling pathways cooperate, we have just begun interbreeding selected erbB-4 lines with both the MMTV/neu and MMTV/NDF strains. The results of these transgenic model that intercrossing experiments should provide important insight into the importance of the erbB-4 signaling pathway in neu mediated tumorigenesis.

CONCLUSIONS AND FUTURE DIRECTIONS:

As a first step in our analyses of the role of EGFR family members in the induction of mammary tumors, we have derived a transgenic model expressing a constitutively active version of neu in the mammary glands (NDL strain). Unlike the parental MMTV/wild type strain which developed focal mammary tumors with a variable onset, the NDL strain developed multifocal mammary tumors in a relatively synchronous fashion (Figure 1). The rapid onset of tumors in these strains was further correlated with high levels of expression of the NDL transgene (Figure 2) and elevated levels of tyrosine phosphorylated Neu protein (Figure 3). In addition, to the high incidence of mammary tumors in these strains, 100% of the tumor bearing female NDL mice develop metastatic lesions to the lung (Siegel and Muller, unpublished observations).

Our previous Army-sponsored research had established that co-activation of Neu and EGFR signaling pathways resulted in the synergistic transformation of the mammary epithelium (Muller et al., 1996, see appended manuscript). More recently, we have attempted to assess whether a functional EGFR is required for the induction of mammary tumors by Neu. To this end we have intercrossed the NDL strains to a naturally occurring mouse mutant which encodes a catalytically impaired EGFR (Waved-2 mouse). Although the results are preliminary, analyses of initial sets of animals carrying the NDL transgene on either a Waved-2 heterozygous or Waved-2 homozygous transgene revealed that both sets of animals have developed mammary tumors (Figure 4A). However while the tumors appeared to arise within a similar time period in these animals, the tumors in the Waved-2 homozygous background grew much more slowly than those which arose in the Waved-2 heterozygous background (Figure 4A). The difference in the apparent growth rate of these tumors cannot be accounted for by differences in the levels of the NDL transcripts since both categories of tumors expressed equivalent levels of NDL transcript (Figure 4B). These results suggest that although a catalytically active EGFR is dispensable for tumor induction by activated its activity may facilitate tumor growth.

Our immediate future objectives with the NDL/Waved-2 interbreeds is to validate these observations with greater numbers of animals. These animals have been generated and should be developing mammary tumors within the next few months. In the course of these analyses we plan to monitor tumor growth by measuring tumor volume by calipers in tumors arising either in the Waved-2 heterozygous or homozygous Waved-2 background. In addition to theses phenotypic measurements, we plan also to continue to assess the levels of transgene in these different expressions by RNase protection analyses to ensure that the observed differential growth effect is not a reflection of differences in levels of transgene expression. Moreover, we plan to assess the state of tyrosine phosphorylation of Neu and EGFR in these to determine if the activity of these EGFR receptor family members is affected by Waved-2 mutation. We anticipate, as previously reported, that EGFR activity should be severely impaired in the Waved-2 homozygous background (Lutteke et al., 1994) but Neu activity should remain unaffected. Both these biochemical and phenotypic analyses should provide important insight into the role of EGFR in Neu mediated tumorigenesis.

The observation that EGFR activity is not absolutely required for the induction of a mammary tumor by activated Neu but may influence tumor growth has important implications in understanding the interaction of these EGFR family members. One possible explanation for these observations is that although the EGFR is catalytically inactive, it can partially signal through transphosphorylation by activated Neu. Indeed it has been demonstrated that a catalytically inactive Neu can be tyrosine phosphorylated by activated EGFR (Qian et al., 1994). Consistent with the view that the Waved-2 mutation is not completely impaired in EGFR signaling, is the observation that in contrast to the viable Waved-2 mutation, a null mutation in EGFR in mice leads to perinatal lethality (Threadgill et al., 1995). Thus this catalytic inactive EGFR receptor still retains some degree of biological activity.

Nonetheless the preliminary data does suggest that the catalytic activity of EGFR does appear to influence tumor growth in the NDL mice. These observations suggest that certain direct substrates of EGFR may participate in Neu induced tumorigenesis. Therefore another important goal of these studies will be to test whether there is a difference in substrate tyrosine phosphorylation between the Neu expressing Waved-2 heterozygous and Waved-2 homozygous tumors. To accomplish this objective we initially will perform immunoblot analyses with antiphosphophotyrosine antibodies on these tumor lysates. These analyses may lead to the identification of key EGFR specific substrates involved in Neu mediated tumorigenesis. The results of these studies will hopefully unveil possible new therapeutic approaches in treating human breast cancers that express elevated levels of these EGFR families.

Another major focus of the upcoming year is the identification of region in Neu involved in the differential binding of c-Src. Based on our analysis of Src interaction with chimeric receptors, the sequences present in the Neu catalytic domain appear to be critical in conferring the capacity to bind c-Src (Figure 7). Furthermore the data suggest that the sequences present in the carboxyl terminus of the Neu catalytic domain are required for Src binding. Because previous studies had demonstrated that Src binding to Neu required a specific tyrosine phosphorylation site (Muthuswamy

and Muller, 1995b), we inspected this region for potential consensus Src binding sites. The results of these analyses revealed that one tyrosine residue within this region conformed to the c-Src consensus binding site (YDGIP, tyrosine 891). In order to assess whether this tyrosine residue is required for c-Src binding, we plan to mutate this site to a phenylalanine residue and assess whether this mutant neu receptor retains its ability to bind c-Src.

However, because of the proximity of this tyrosine residue to the catalytic domain, it is conceivable that this phenylalanine substitution may interfere with the intrinsic catalytic activity of neu rendering the interpretation of this data difficult. Indeed, inactivation the Platelet Derived Growth Factor Receptor (PDGFR) c-Src binding site leads to dramatic impairment of PDGFR associated tyrosine kinase activity. If this proves to be the case, we will attempt to assess whether we can interfere with c-Src binding to Neu with tyrosine phosphorylated peptide corresponding to the tyrosine phosphorylated Src binding site. Finally we will assess the biological consequences of inactivation of the c-Src binding site on Neu by assessing whether Src binding to Neu is required for its capacity to collaborate with the EGFR in its capacity to transform. This can be accomplished by assessing whether the different chimeric Neu/EGFR receptors (Figure 7) or a mutant neu receptor lacking the c-src binding site (i.e. Tyr 891 to Phe) to collaborate with EGFR to transform rat-1 fibroblasts. Alternatively, we can indirectly assess the role of c-Src in EGFR/Neu mediated transformation by use of the competitor tyrosine phosphorylated c-Src binding peptide. Depending on the outcome of these studies we plan to extend these observation to established mammary tumor cell lines co-expressing EGFR and Neu. The results of these proposed studies could have important therapeutic implications in the treatment of human breast cancers that overexpress these EGFR families.

In addition to these proposed studies, we plan to continue our studies directed to assess the role of the other members of the EGFR family in mammary tumorigenesis. To this end we have in the last year generated transgenic mice that express the erbB-4 proto-oncogene in the mammary epithelium. (Figure 10). We are currently monitoring these animals for the occurrence of mammary tumors. One possible outcome of these experiments is that like many of the tumors arising in MMTV/wild type neu mice, somatic activating mutations in erbB-4 may be required before tumors arise in the MMTV/erbB-4 strains. In this regard, the region deleted in neu demonstrates a high degree of conservation in the other EGFR family members including erbB-4 (Siegel and Muller, 1996, see appended manuscript #4). Given the close homology between these receptors, we will screen tumors arising in the erbB-4 mice for the presence of similar activating mutations.

Because we have previously demonstrated that Neu induced tumors express elevated levels of erbB-4 (Muthuswamy and Muller, unpublished observations), we are currently interbreeding MMTV/wild type neu mice with one of the MMTV/erbB-4 strains (line #6) to derive bigenic mice that co-express erbB-4 and Neu in the mammary epithelium. If erbB-4 and neu can collaborate to transform the mammary epithelium, we anticipate that bigenic mice should develop tumors with accelerated kinetics relative to either parental strain. If this proves to be the case, we will assess the state of tyrosine phosphorylation of Neu and erbB-4 in these tumors. In addition, we will ascertain

whether the observed cooperativity between erbB-4 and Neu is due to its capacity to form heterodimers between these two receptors. These experiments should shed important insight into the function of erbB-4 in Neu mediated tumorigenesis.

In addition to testing the whether erbB-4 and Neu can collaborate, we plan to test whether co-expression of erbB-4 and its cognate ligand NDF can result in the synergistic transformation of the mammary epithelium. Indeed, several studies have suggested those mammary tumor cells co-expressing NDF and erbB-4 are highly tumorigenic due to the establishment of this autocrine growth factor loop (Salomon, et al., 1995). Moreover overexpression of the NDF ligand alone n the mammary epithelium results in the induction of mammary tumors (Krane and Leder, 1996). To accomplish this research objective, we have obtained the recently published MMTV/NDF strains (Krane and Leder, 1996) and have interbred them with our MMTV/erbB-4 strains. We anticipate that co-expression of NDF and its receptor should result in acceleration of mammary tumors in bigenic transgene carriers compared to either parental strain. Tumors arising in these mice will be subjected to biochemical and analyses to ensure that both transgenes are co-expressed in the mammary epithelium. The results of these experiments should allow us to test the importance of this autocrine growth loop in the induction of mammary cancers.

Our final research objectives for the coming year will involve the establishment of transgenic strains expressing either the EGFR and erbB-3 in the mammary epithelium. To accomplish this we are currently microinjecting in one cell mouse zygotes recombinant plasmids comprising MMTV driven erbB-3 and EGFR receptor constructs. These experiments should provide direct evidence for the involvement of these other EGFR family members in mammary tumorigenesis.

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APPENDIX 1

FIGURES AND LEGENDS 1-10

Figure 1: Kinetics of tumor occurrence in wild-type *neu* and NDL1 transgenic mouse strains. Comparison of the kinetics of tumor formation between virgin females of the N202 (wild-type) and NDL1-2 (**N**eu **De**letion) lines. The age at which 50% of the animals were found to have tumors (T50) and the number of mice examined (n) is indicated.

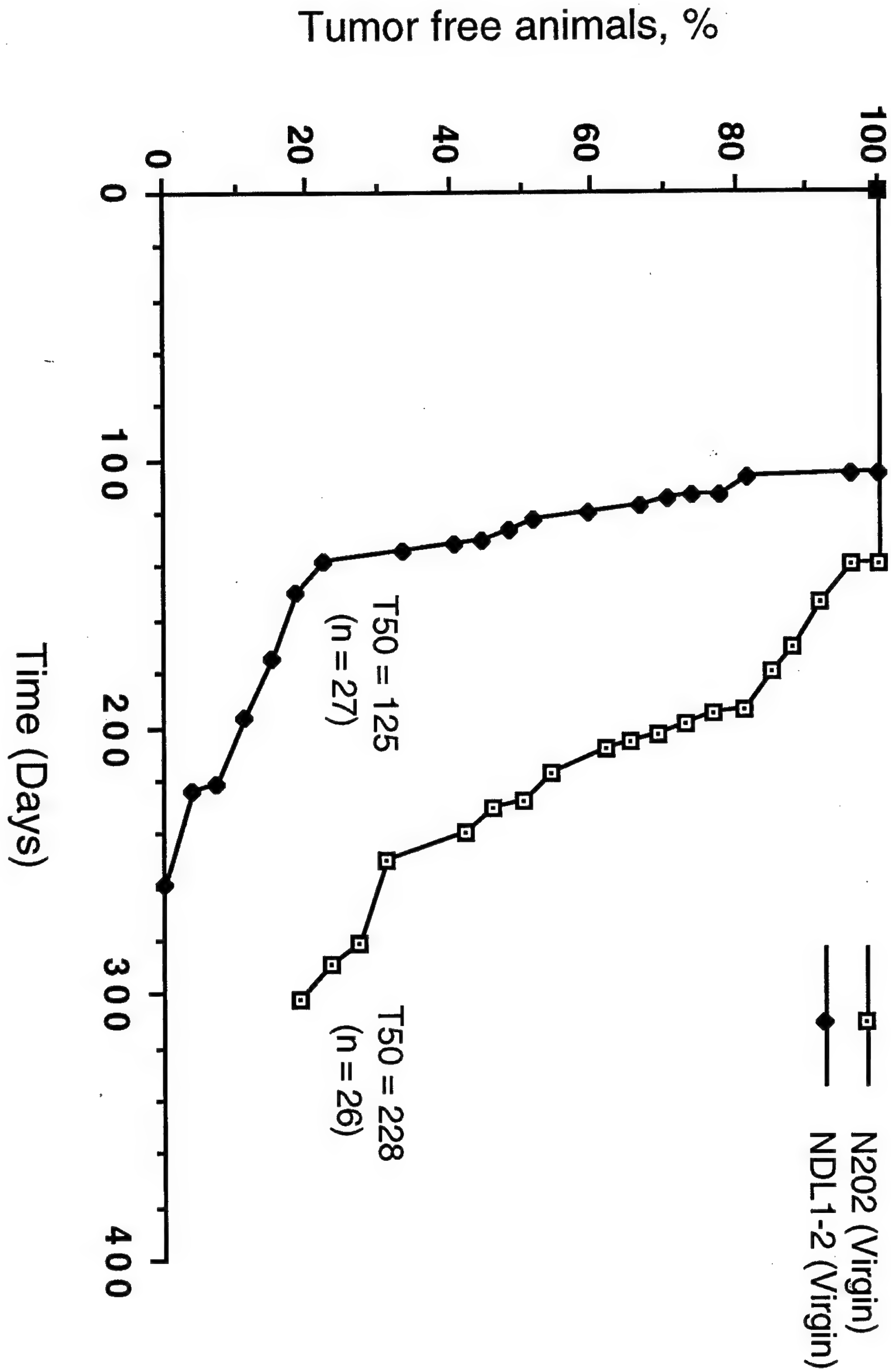

Tumor free animals, %
0
20
40
60
80
100
0
100
200
300
400
Time (Days)
T50 = 125
(n = 27)
T50 = 228
(n = 26)
N202 (Virgin)
NDL1-2 (Virgin)

Figure 2: Structure of transgene and tissue specificity of transgene expression. (A) Diagrammatic representation of the MMTV/neu deletion transgene. The unshaded region represents Bluescript vector sequences, the striped region indicates the mouse mammary tumor virus long terminal repeat sequences, the filled in area represents the *neu* cDNA harboring a 12 amino acid deletion, and the grey region indicates transcriptional processing sequences derived from the SV40 early transcription unit. Limited protein sequence located above the schematic defines the precise nature and location of the deletion. The 285 nt riboprobe utilized in the RNase protections shown in parts B and C of this figure is indicated. (B) RNA transcripts corresponding to the MMTV/NDL1 transgene in various organs of a female mouse from the NDL1-2 line. Tissues were derived from a multiparous female (#7985) at 118 days of age. The antisense riboprobe protects a 285 nt fragment indicated by SPA and an arrow. Also shown is an internal control riboprobe directed against the phosphoglycerate kinase gene. The PGK-1 riboprobe protects a fragment of 124 nt and is marked by PGK-1 and an arrow. (C) RNA transcripts corresponding to the MMTV/NDL1 transgene in various organs of a male mouse from the NDL1-2 line. Tissues were derived from a male animal (#7991) at 185 days of age. The riboprobes used for this RNase protection analysis are as described in (B). The numbers on the left indicate the size of the molecular weight markers in nucleotides.

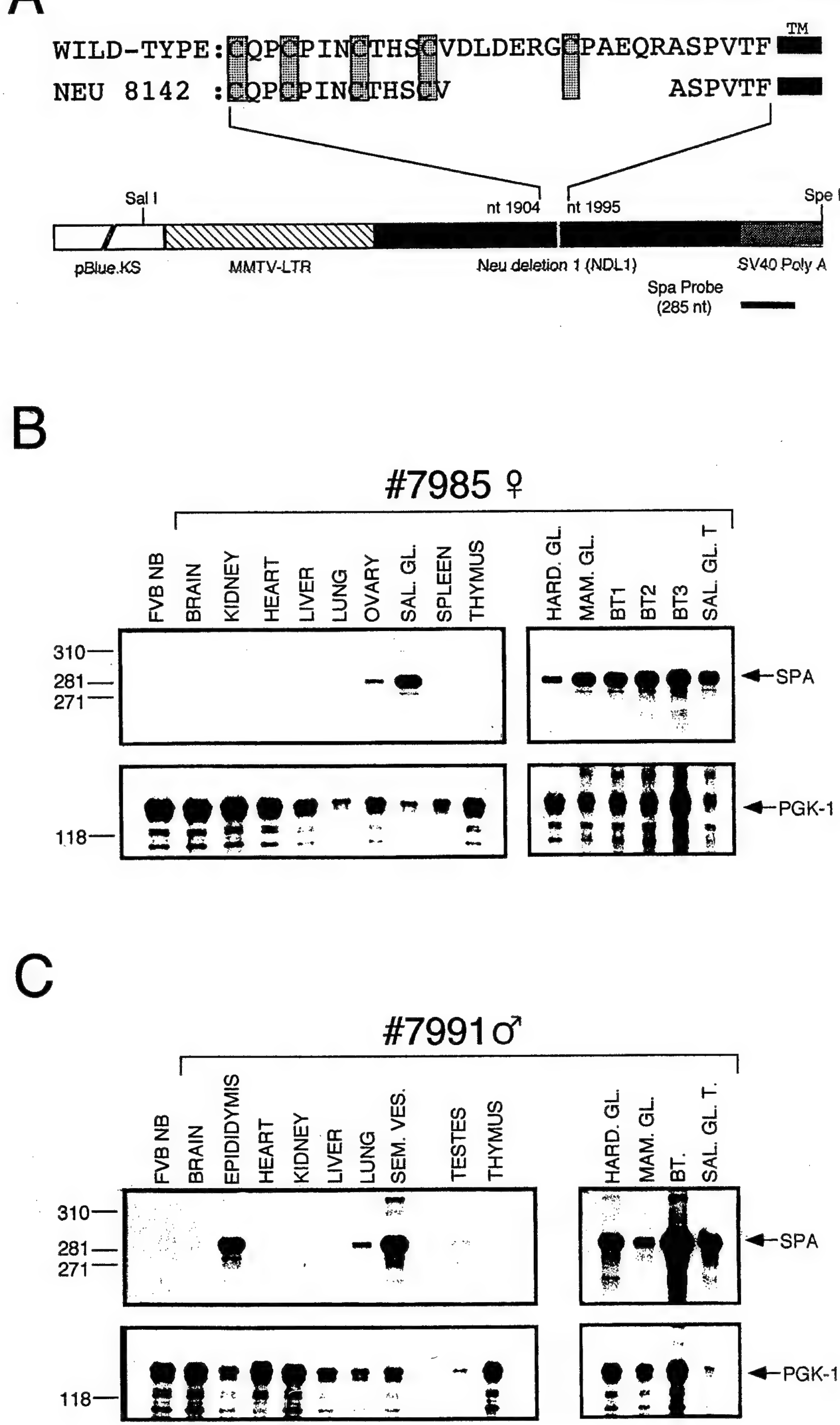
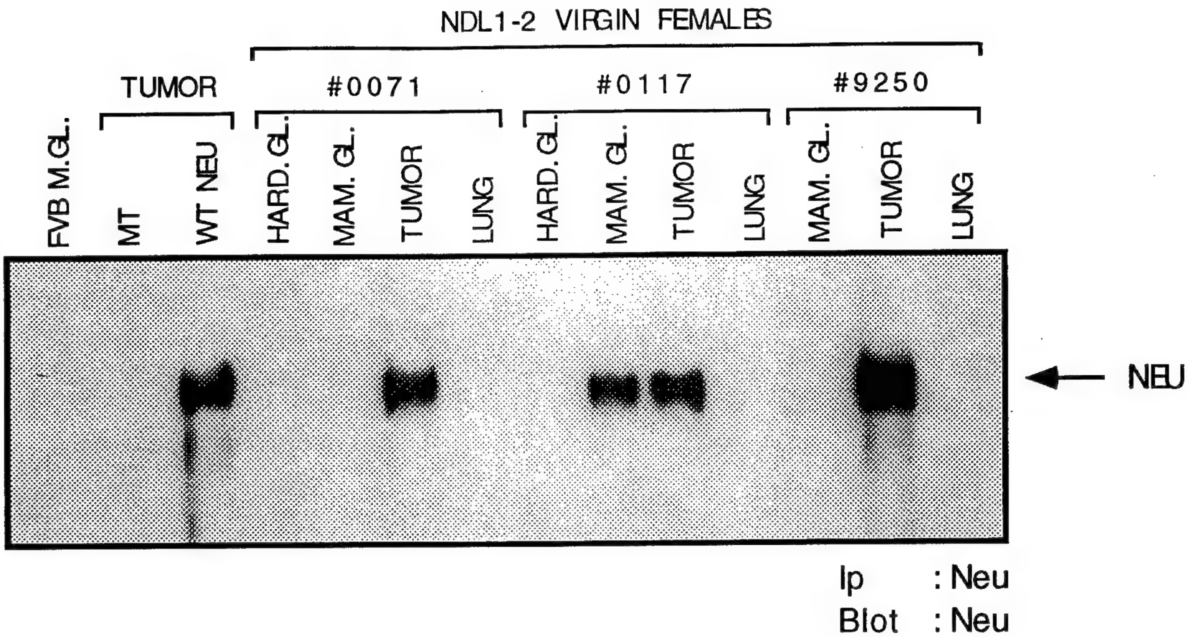

A
TM
WILD-TYPE:CQPCPINCTHSCVDLDERGCPAEQRASPVTF
NEU 8142 :CQPCPINCTHSCV ASPVTF
Sal I
nt 1904
nt 1995
Spe I
pBlue.KS
MMTV-LTR
Neu deletion 1 (NDL1)
SV40 Poly A
Spa Probe
(285 nt)
B
#7985 ♀
FVB NB
BRAIN
KIDNEY
HEART
LIVER
LUNG
OVARY
SAL. GL.
SPLEEN
THYMUS
HARD. GL.
MAM. GL.
BT1
BT2
BT3
SAL. GL. T
310
281
271
118
SPA
PGK-1
C
#7991 ♂
FVB NB
BRAIN
EPIDIDYMIS
HEART
KIDNEY
LIVER
LUNG
SEM. VES.
TESTES
THYMUS
HARD. GL.
MAM. GL.
BT.
SAL. GL. T.
310
281
271
118
SPA
PGK-1

Figure 3: Expression of tyrosine phosphorylated Neu in tumors obtained from MMTV/NDL1 mice. (A) Lysates from the indicated tissues were made from virgin females of the NDL1-2 line, control tissues were included from wild-type FVB as well as MMTV/mT and MMTV/wild-type *neu* transgenic animals. Neu was immunoprecipitated from the lysates (Ab 7.16.4) and 50% of the immunoprecipitate was subjected to immunoblot analysis with a Neu-specific antibody (Ab3). The position of Neu is marked by an arrow. (C) The remaining 50% of the immunoprecipitate was subjected to immunoblot analysis with phosphotyrosine-specific antibody (PY20). The position of tyrosine phosphorylated Neu is indicated by the arrow.

A


NDL1-2 VIRGIN FEMALES
TUMOR
#0071
#0117
#9250
FVB M.GL.
MT
WT NEU
HARD. GL.
MAM. GL.
TUMOR
LUNG
HARD. GL.
MAM. GL.
TUMOR
LUNG
MAM. GL.
TUMOR
LUNG
NEU
Ip : Neu
Blot : Neu

B

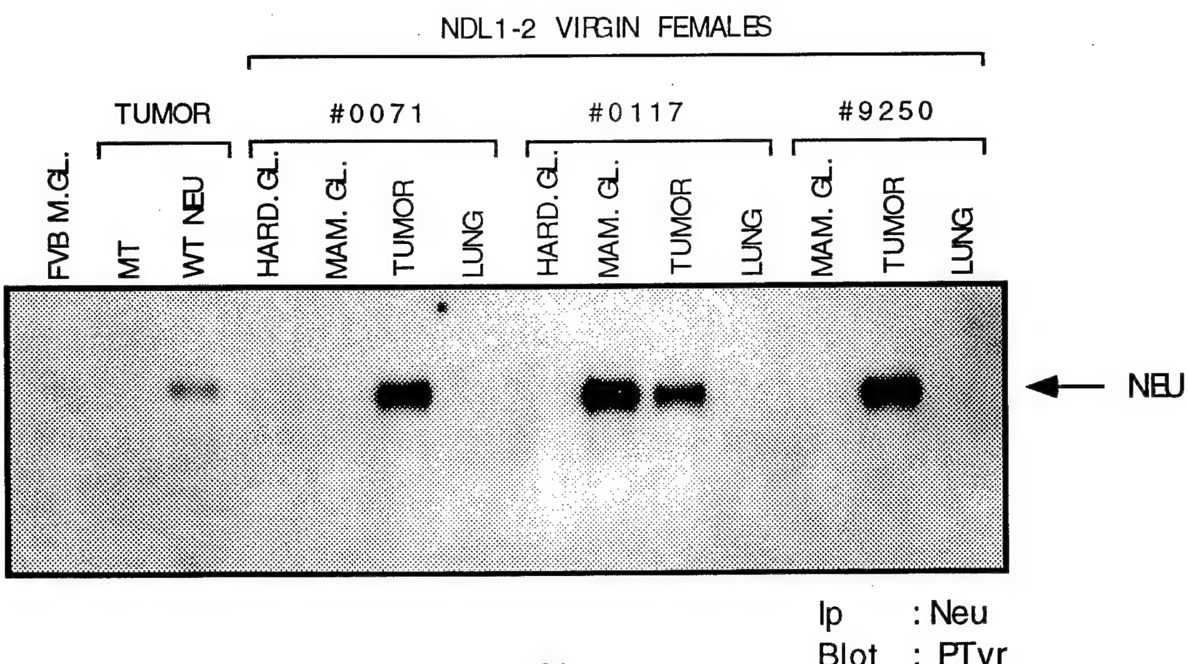

NDL1-2 VIRGIN FEMALES
TUMOR
#0071
#0117
#9250
FVB M.GL.
MT
WT NEU
HARD. GL.
MAM. GL.
TUMOR
LUNG
HARD. GL.
MAM. GL.
TUMOR
LUNG
MAM. GL.
TUMOR
LUNG
NEU
Ip : Neu
Blot : PTyr

Figure 4: Mammary tumor formation in MMTV/ndl-1 transgenic virgin female mice in a waved-2 genetic background. (A) Phenotypic comparison of ndl-1 transgenic mice heterozygous or homozygous for the wa-2 mutation. Note the presence of a large tumor in the mammary gland of the wa-2/+ mouse. Both mice are 231 days of age. (B) RNase protection analysis of expression of ndl-1 transcripts in wa-2/+ and wa-2/wa-2 mammary epithelium. RNA was isolated from tissue derived from each of the above mice and hybridized against the anitsense rat neu riboprobe. An antisense probe directed against the mouse phosphoglycerate kinase gene (pgk-1) was used as control for equal loading of RNA on the gel.

A

ndl-1/+; wa-2/+

ndl-1/+; wa-2/wa-2

B

ndl-1/+; wa-2/+

ndl-1/+; wa-2/wa-2

NDL1 Transcript

PGK-1

1 2

Figure. 5. Schematic representation of Neu receptor kinase mutants. **(A)** The structure of Neu and activated Neu (Neu ^{NT}), containing the transmembrane mutation V664E, are depicted. Indicated are two cysteine rich domains (shaded), a single transmembrane domain (black) and the tyrosine kinase domain (striped). Autophosphorylation sites at tyrosine residues 1028 (site A), 1144 (site B), 1201 (site C), 1226/7(site D) and 1253 (site E) are indicated (). **(B)** Indicated tyrosine residues were converted to phenylalanine residues (). NT-CT1 carries a stop codon immediately following the methionine codon at 1005 and NT-CT2 is a frame shift deletion mutant terminating with the sequences 1005M<u>HGQYLLPFTAGR</u>, where the underlined amino acids differ from that of Neu. **(C)** NT-NYPD contains mutations at each of the indicated autophosphorylation sites. Add-back mutants derived from NT-NYPD contain single autophosphorylation sites and phenylalanine residues at the four remaining sites. All mutants were derived from Neu^{NT}.

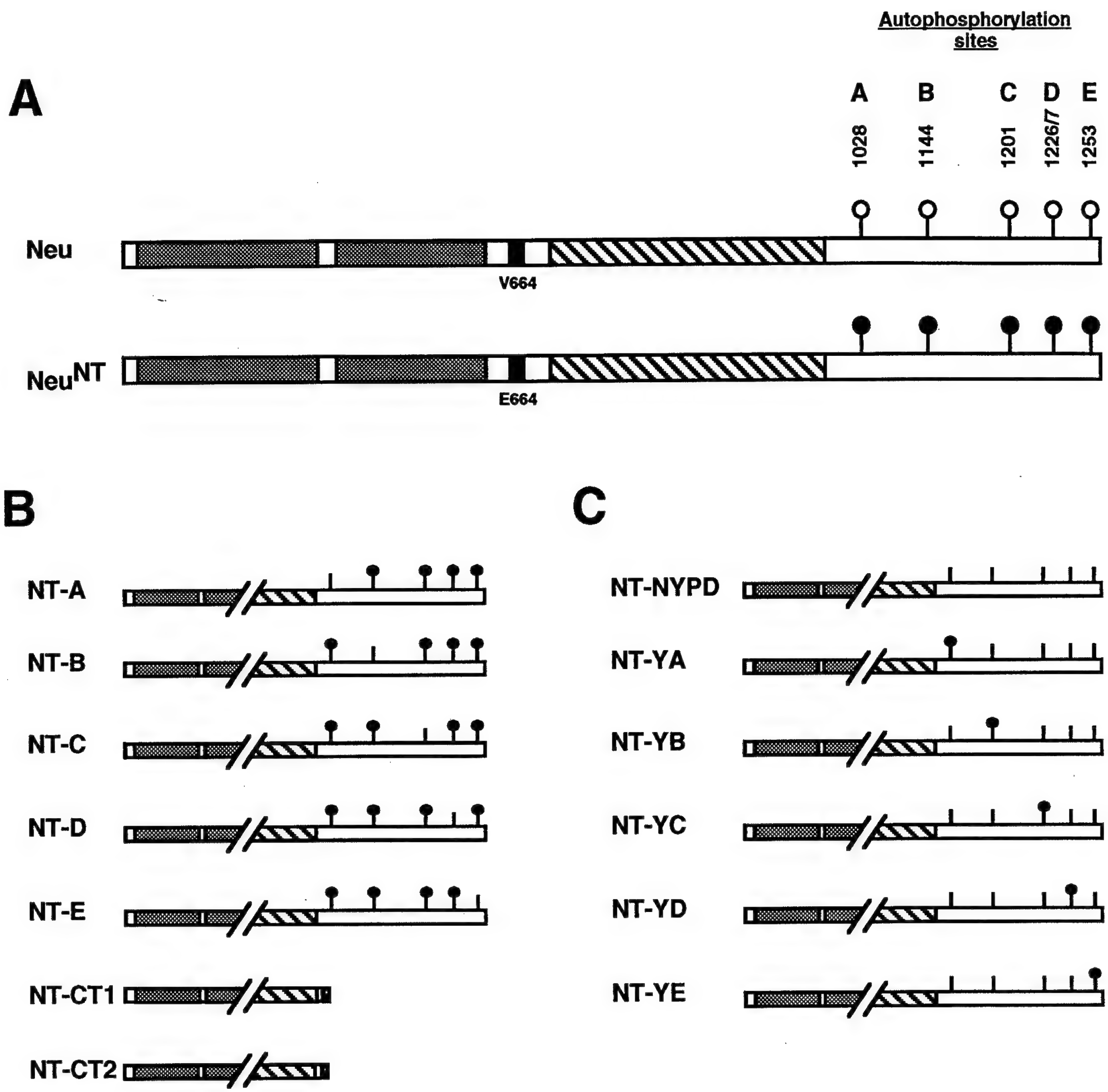

Autophosphorylation sites
A
A B C D E
1028 1144 1201 1226/7 1253
Neu
V664
NeuNT
E664
B
NT-A
NT-B
NT-C
NT-D
NT-E
NT-CT1
NT-CT2
C
NT-NYPD
NT-YA
NT-YB
NT-YC
NT-YD
NT-YE

Figure 6. Src associates with Neu through a site(s) which differs from that of the identified carboxyl-terminal autophosphorylation sites. (**A**) Neu was immunoprecipitated from 1.5 mg of protein lysate obtained from the indicated Rat1-derived cell lines. Two thirds of each immunoprecipitate was electrophoresed on an SDS-8.5% gel, transferred to a PVDF membrane and probed with a ^{32}P-radiolabeled GST-Src-SH2 domain fusion protein. (**B**) The remaining portion of each immunoprecipitate was subjected to immunoblot analyses with anti-phosphotyrosine monoclonal antibodies (mABs). (**C**) Src was immunoprecipitated from 1.5 mg of protein lysate and immunoprecipitates were subjected to immunoblot analyses with anti-Neu mABs. Neu was immunoprecipitated from 0.5 mg of the same protein lysate and immunoprecipitates were probed with anti-phosphotyrosine mABs.

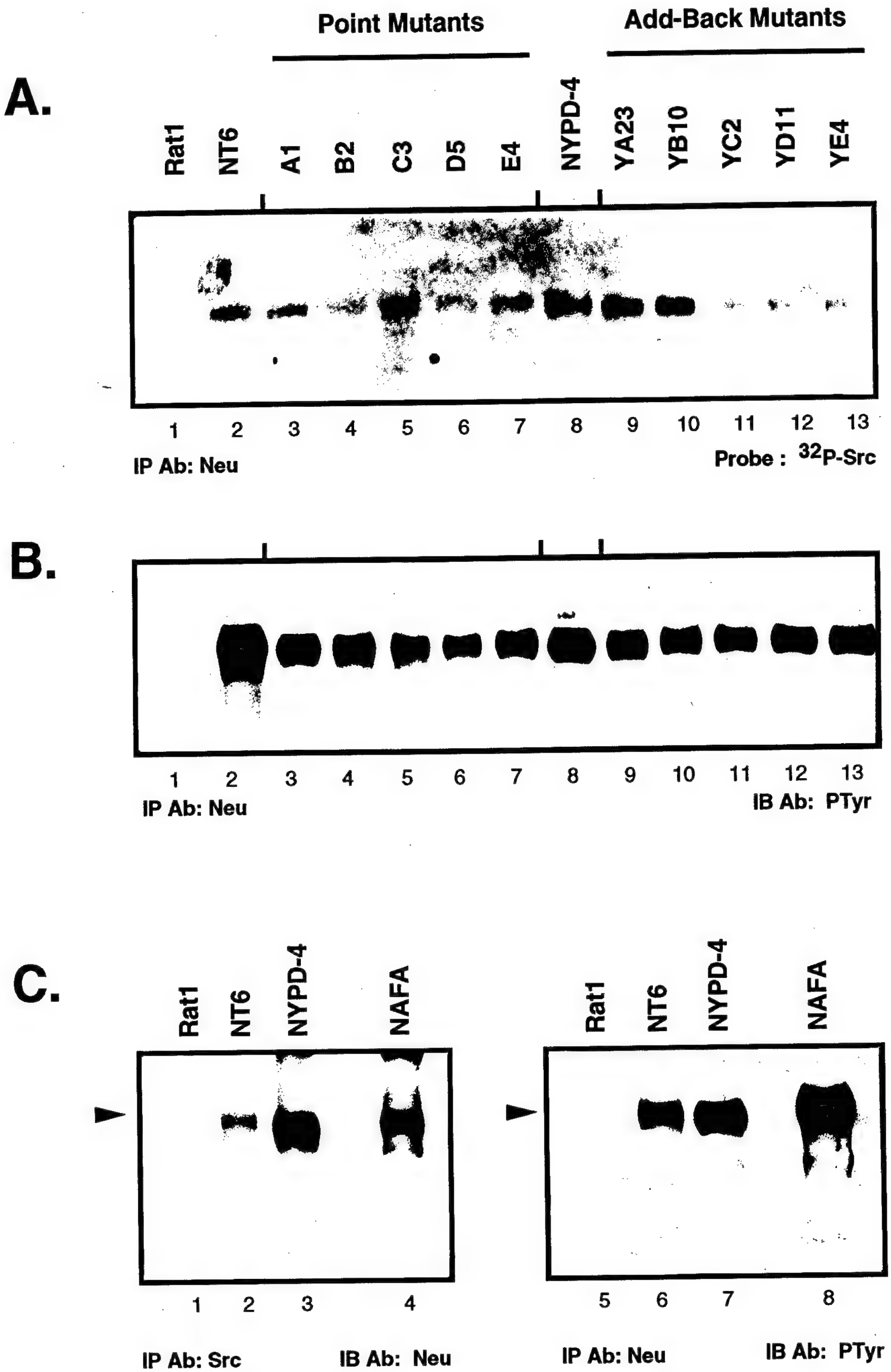

Point Mutants
Add-Back Mutants
A.
Rat1
NT6
A1
B2
C3
D5
E4
NYPD-4
YA23
YB10
YC2
YD11
YE4
1 2 3 4 5 6 7 8 9 10 11 12 13
IP Ab: Neu
Probe : 32P-Src
B.
1 2 3 4 5 6 7 8 9 10 11 12 13
IP Ab: Neu
IB Ab: PTyr
C.
Rat1
NT6
NYPD-4
NAFA
1 2 3 4
IP Ab: Src
IB Ab: Neu
Rat1
NT6
NYPD-4
NAFA
5 6 7 8
IP Ab: Neu
IB Ab: PTyr

Figure 7. Chimeric constructs. Diagrammatic representation of EGFR/c-ErbB2 chimeric constructs. Expression of wild type EGFR and the chimeric receptors are driven by the molony murine leukemia virus LTR. Stable cell lines in Rat 1 fibroblasts were established by calcium phoshpate co-transfection using pgkPuromycin as a selectable marker.

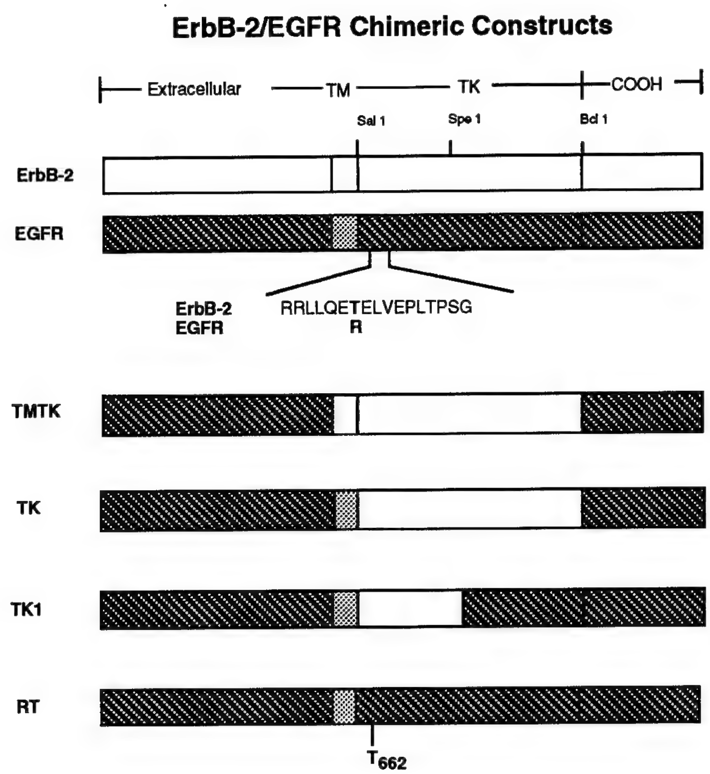

ErbB-2/EGFR Chimeric Constructs
Extracellular
TM
TK
COOH
Sal 1
Spe 1
Bcl 1
ErbB-2
EGFR
ErbB-2
EGFR
RRLLQETELVEPLTPSG
R
TMTK
TK
TK1
RT
T662

Figure 8. Stable Rat-1 cell lines derived for high level expression of EGFR/ErbB2 chimeras. Western analysis of stable cell lines derived via calcium phosphate transfection were lysed in MTNE lysis buffer with appropriate proteases and phosphatase inhibitors. Confluent 100mm plates were lysed for 30 minutes on ice and cleared by centrifugation. 100 ug of protein was boiled for 5 minutes in 2x SDS loading buffer and fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene diflouride membrane (PVDF) (Millipore). Membranes were blocked with 3% skim milk/PBS and probed with anti-EGFR (Transduction Laboratories) or anti-Neu (7.16.4). Molecular weight markers are shown in kDa. N202 is mammary tissue derived from a Neu transgenic mouse generated by Guy *et. al.* (1989). RHER is a Rat 1 fibroblast line expressing high levels of the EGF receptor.

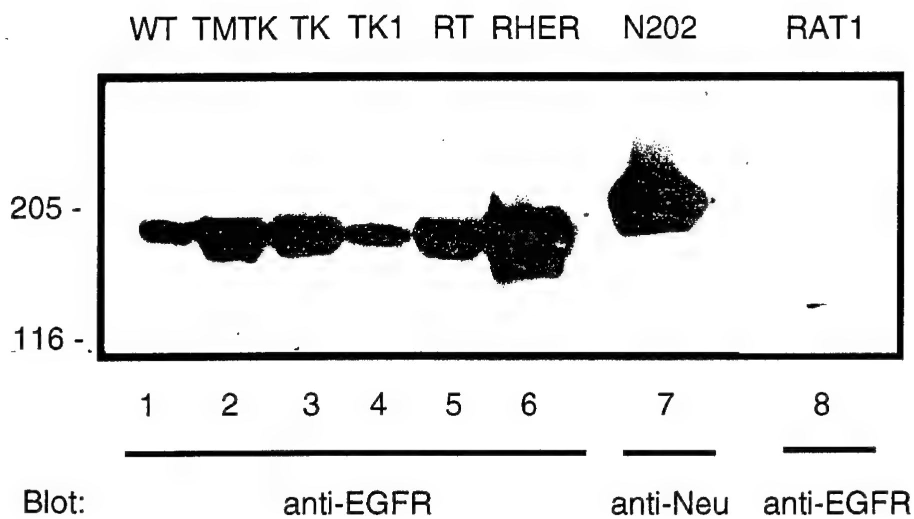
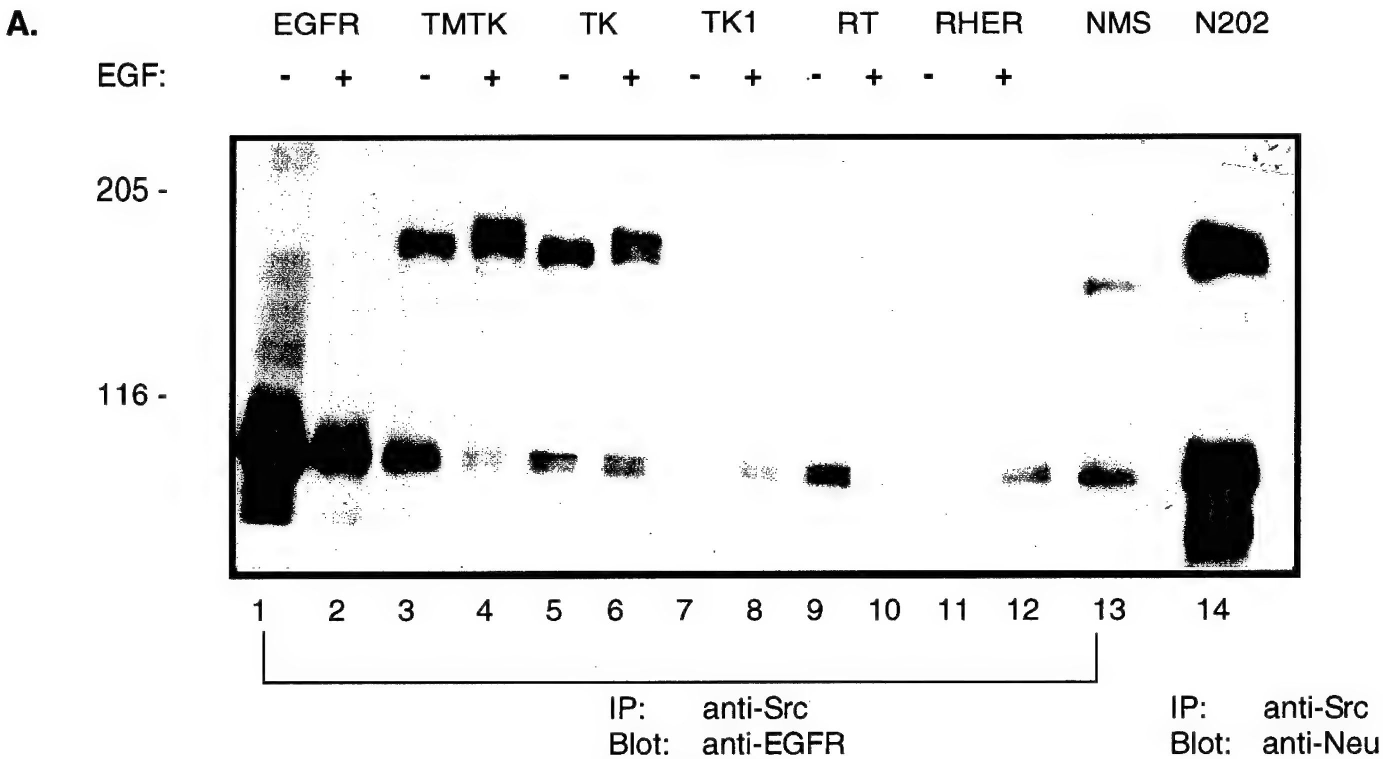

WT
TMTK
TK
TK1
RT
RHER
N202
RAT1
205 -
116 -
1
2
3
4
5
6
7
8
Blot:
anti-EGFR
anti-Neu
anti-EGFR

Figure 9A. Src co-immunoprecipitates specifically with TMTK and TK. Amount of association is dependent on the level of chimeric receptor tyrosine phosphorylation. Positive control is N202 mammary tumor tissue. Co-immunoprecipitation analysis of stable cell lines on confluent (150mm) plates were performed following EGF stimulation (100ng/ml) for 5 minutes. All plates were washed twice in ice cold PBS with 1mM sodium orthovanadate and lysed on ice with 0.7% 3-{(cholamidopropyl)-dimethyl-ammonio}-1-propaneosulphonate (CHAPS) lysis buffer (50 mM Tris HCl pH 8.0, 0.7% CHAPS, 50mM NaCl, 1 mM sodium orthovanadate, 10ug/ml leupeptin, 10ug/ml aprotinin). Immunoprecipitations were performed by incubating 1.5 mg of total cell lysate with 2-3 ug of anti-Src (7D10, Quality Biotech) or NMS and 40 ul of protein G separose for 3 hours rotating at $4^{o}C$ and subsequently washed 4 times with 0.7% CHAPS lysis buffer. Samples were boiled for 5 minutes in 40 ul of 1x SDS loading buffer and fractionated by SDS-PAGE. Immunoblotting of PVDF membranes with anti-EGFR (Transduction Laboratories) or anti-Neu (7.16.4) was performed via standard techniques. Molecular weight markers are shown in kDa.

Figure 9B. Chimeric receptors undergo tyrosine phosphorylation upon EGF stimulation. Following EGF stimulation (100ng/ml) for 5 minutes, IP western analysis of stable cell lines was performed with 500 ug of lysates immunoprecipitated with anti-EGFR or anti-Neu and probed with anti-phosphotyrosine (PY20, Transduction Laboratories). Immunoblotting of PVDF membranes was performed via standard techniques. Molecular weight markers are shown in kDa.


A.
EGFR
TMTK
TK
TK1
RT
RHER
NMS
N202
EGF:
- + - + - + - + - + - +
205 -
116 -
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IP: anti-Src
Blot: anti-EGFR
IP: anti-Src
Blot: anti-Neu

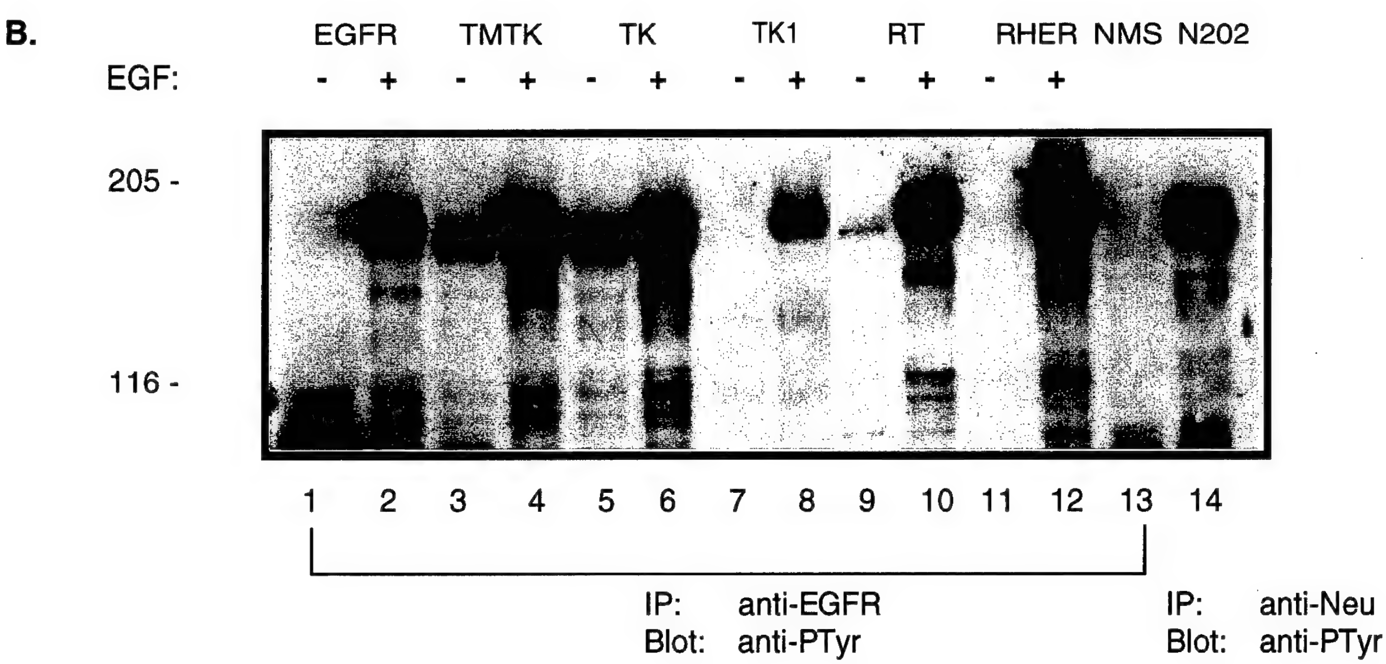
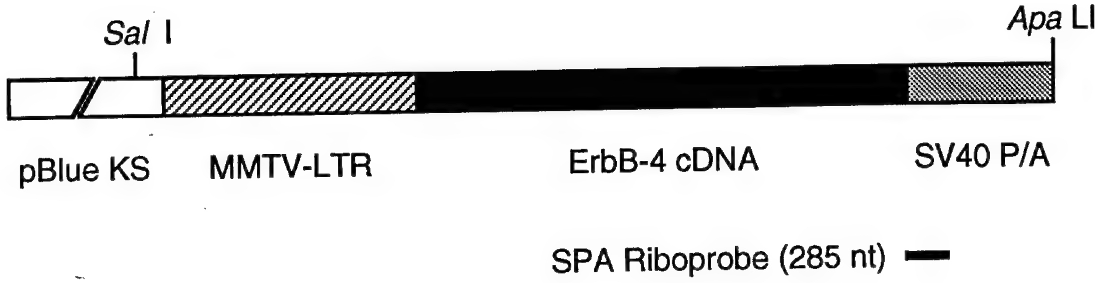
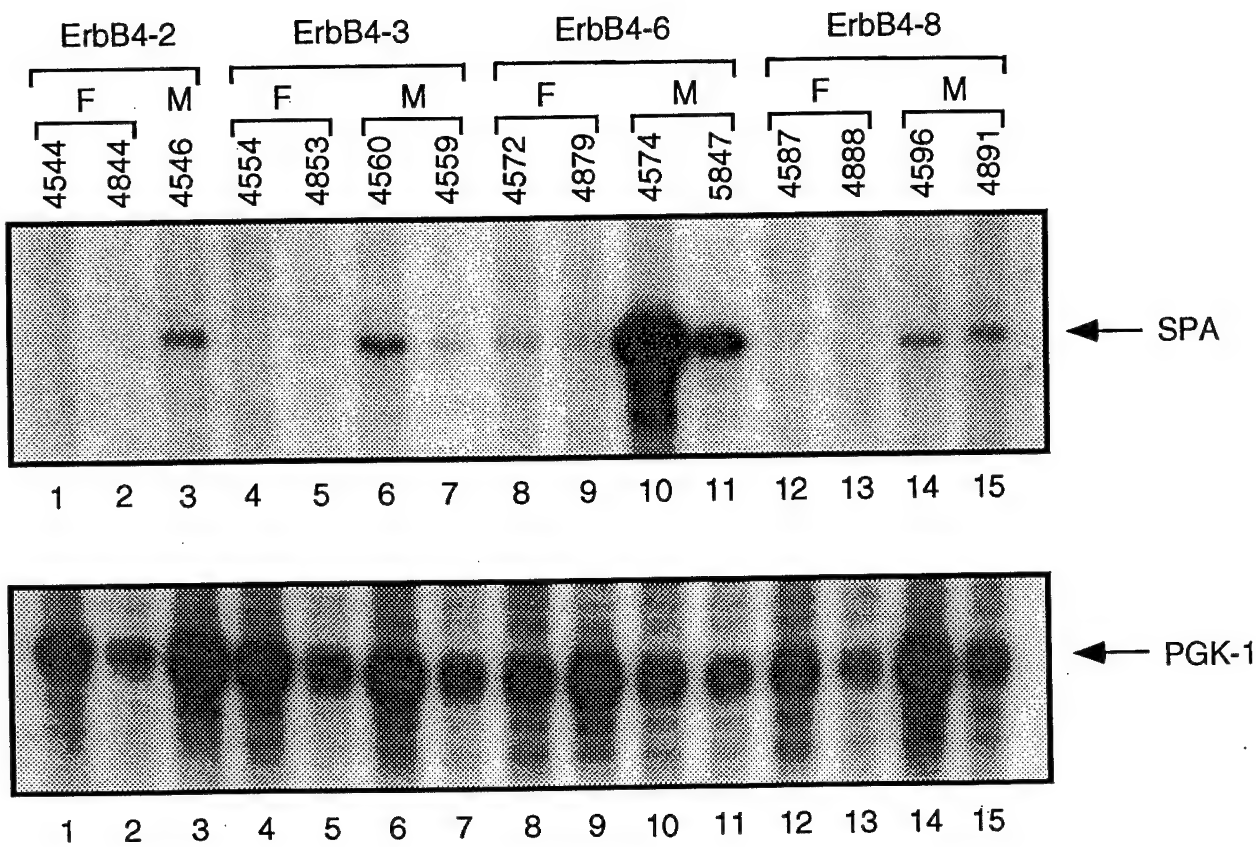

B.
EGFR
TMTK
TK
TK1
RT
RHER
NMS
N202
EGF:
- + - + - + - + - + - +
205 -
116 -
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IP: anti-EGFR
Blot: anti-PTyr
IP: anti-Neu
Blot: anti-PTyr

Figure 10: Structure of transgene and transgene expression in several transgenic lines. (A) Diagrammatic representation of the MMTV/erbB-4 transgene. The unshaded region represents Bluescript vector sequences, the striped region indicates the mouse mammary tumor virus long terminal repeat sequences, the filled in area represents the murine erbB-4 cDNA, and the grey region indicates transcriptional processing sequences derived from the SV40 early transcription unit. The 285 nt riboprobe utilized in the RNase protection shown in part B of this figure is indicated. (B) RNA transcripts corresponding to the MMTV/erbB-4 transgene in the mammary gland (females) and testes (male) from various transgenic lines. The first female in each set was a virgin animal while the second female in each set had given birth to one litter. The antisense riboprobe protects a 285 nt fragment indicated by SPA and an arrow. Also shown is an internal control riboprobe directed against the phosphoglycerate kinase gene. The PGK-1 riboprobe protects a fragment of 124 nt and is marked by PGK-1 and an arrow.

A


Sal I
Apa LI
pBlue KS
MMTV-LTR
ErbB-4 cDNA
SV40 P/A
SPA Riboprobe (285 nt)

B


ErbB4-2
ErbB4-3
ErbB4-6
ErbB4-8
F
M
4544
4844
4546
4554
4853
4560
4559
4572
4879
4574
5847
4587
4888
4596
4891
SPA
PGK-1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Muller, William J.
Grant #DAMD17-94-J-4300

APPENDIX 2

MANUSCRIPT #1 - Muller, et al., 1996
#2 - Muthuswamy & Muller, 1995
#3 - Muthuswamy & Muller, 1995
#4 - Siegel and Muller, 1996

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0270-7306/96/$04.00+0

Vol. 16, No. 10

Synergistic Interaction of the Neu Proto-Oncogene Product and Transforming Growth Factor α in the Mammary Epithelium of Transgenic Mice

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WILLIAM J. MULLER,[1,2*] CARLOS L. ARTEAGA,[3,4] SENTHIL K. MUTHUSWAMY,[1,5] PETER M. SIEGEL,[1,5] MARC A. WEBSTER,[1,5] ROBERT D. CARDIFF,[6] KATHERINE S. MEISE,[3] FENG LI,[3] SUSAN A. HALTER,[4] AND ROBERT J. COFFEY[3]

Institute for Molecular Biology and Biotechnology[1] and Departments of Pathology[2] and Biology,[5] McMaster University, Hamilton, Ontario, Canada; Department of Pathology, School of Medicine, University of California at Davis, Davis, California 95616,[6] and Departments of Medicine and Cell Biology[3] Pathology,[4] Veterans Association, Vanderbilt University, Nashville, Tennessee 37232

Veterans Administration Medical Center

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Transgenic mice expressing either the *neu* proto-oncogene or transforming growth factor (TGF-α) in the mammary epithelium develop spontaneous focal mammary tumors that occur after a long latency. Since the epidermal growth factor receptor (EGFR) and Neu are capable of forming heterodimers that are responsive to EGFR ligands such as TGF-α, we examined whether coexpression of TGF-α and Neu in mammary epithelium could cooperate to accelerate the onset of mammary tumors. To test this hypothesis, we interbred separate transgenic strains harboring either a mouse mammary tumor virus/TGF-α or a mouse mammary tumor virus/*neu* transgene to generate bitransgenic mice that coexpress TGF-α and *neu* in the mammary epithelium. Female mice coexpressing TGF-α and *neu* developed multifocal mammary tumors which arose after a significantly shorter latency period than either parental strain alone. The development of these mammary tumors was correlated with the tyrosine phosphorylation of Neu and the recruitment of c-Src to the Neu complex. Immunoprecipitation and immunoblot analyses with EGFR- and Neu-specific antisera, however, failed to detect physical complexes of these two receptors. Taken together, these observations suggest that Neu and TGF-α cooperate in mammary tumorigenesis through a mechanism involving Neu and EGFR transactivation.

The epidermal growth factor receptor (EGFR) family comprises four closely related type 1 receptor tyrosine kinases (RTKs) (EGFR, Neu [erbB-2, HER2], erbB-3 [HER3], and erbB-4 [HER4]) that are receptors for a variety of mitogenic growth factors (36). Enhanced expression of the EGFR family has been implicated in the genesis of human breast cancers. For example, amplification and consequent overexpression of *neu* have been observed in a significant proportion of human breast cancers and appear to be inversely correlated with patient survival (9, 25, 32, 33, 37). More recently, overexpression of the other members of the EGFR family, including EGFR, erbB-3, and erbB-4, has also been implicated in the pathogenesis of human breast cancer (15, 16, 26, 27).

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The activity of these EGFR family members can also be affected by expression of a variety of specific ligands for these RTKs. For example, Neu is a substrate of the activated EGFR following stimulation of cells with EGF or transforming growth factor α (TGF-α) (1, 8, 13, 35). Although Neu shares homology with the EGFR, Neu does not bind these EGF ligands. Rather, the observed tyrosine phosphorylation of Neu by the EGFR is thought to be mediated by heterodimerization and/or transactivation between Neu and EGFR, resulting in a high-affinity receptor for these EGFR ligands (8, 39). Consistent with these observations, coexpression of Neu and EGFR results in efficient transformation of fibroblasts in vitro (14). Moreover, elevated expression of both Neu and EGFR can be detected in primary human breast cancers (16). Taken together, these observations suggest that these two closely related RTKs may collaborate in mammary tumorigenesis.

Direct evidence of the involvement of EGFR family members in the induction of mammary tumors derives from observations made with transgenic mice expressing *neu* in the mammary epithelium (4, 10, 21). High-level expression of a constitutively active form of *neu* bearing a point mutation in the transmembrane domain (3) resulted in the development of nonstochastic, multifocal mammary tumors that affected every female carrier (21). In contrast, expression of the wild-type *neu* proto-oncogene in the mammary epithelium of transgenic mice resulted in the focal development of mammary tumors that arose after long latency (10). Interestingly, induction of mammary tumors in wild-type *neu* transgenic mice correlated with the frequent occurrence of activating mutations in the *neu* transgene (31). Thus, activation of the Neu RTK appears to be a pivotal step in the induction of mammary tumors in these mice.

Additional evidence implicating the EGFR family in mammary tumorigenesis derives from observations made with transgenic strains expressing an EGFR-specific ligand, TGF-α, in the mammary epithelium. Mammary gland-targeted expression of TGF-α in various transgenic strains results in the development of mammary epithelial hyperplasias that progress to focal mammary tumors after a long latency, as in wild-type *neu* transgenic mice (11, 18, 29). In mouse mammary tumor virus (MMTV)/TGF-α transgenic mice, increased expression of EGFR was observed in mammary tumors compared with adjacent, histologically normal tissue (18).

Given the potential of Neu and TGF-α (along with enhanced EGFR expression) to cooperate through a mechanism

* Corresponding author.

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of receptor transactivation, we were interested in determining whether coexpression of *neu* and TGF-α could accelerate the induction of mammary tumors in vivo. To accomplish this, separate strains of transgenic mice carrying either an MMTV/*neu* or an MMTV/TGF-α transgene were interbred to generate dual carriers that coexpressed both *neu* and TGF-α in the mammary epithelium. The bigenic animals developed multifocal mammary tumors within a significantly shorter latency period than either parental strain alone. The induction of mammary tumors in these strains was further correlated to tyrosine phosphorylation of Neu and the recruitment of the c-Src tyrosine kinase to this complex, even in the absence of *neu* mutations. Cross-linking studies and reciprocal EGFR and Neu immunoprecipitations, however, did not demonstrate a physical association between EGFR and Neu in bigenic mice. Although these negative data do not disprove a transient physical association between these two receptors, the evidence favors receptor transactivation as the mechanism responsible for
* the observed cooperativity between EGFR and *neu* in mam- Neu (protein) mary tumorigenesis. Taken together, these data argue that coexpression of TGF-α and *neu* can act synergistically to transform the mammary epithelium.

MATERIALS AND METHODS

DNA constructs and generation of transgenic mice. The plasmid used to generate the antisense *neu* riboprobe was constructed by inserting an *Sma*I-*Xba*I fragment (nucleotides 1684 to 2332) into pSL301 (Invitrogen) (31). The phosphoglycerate kinase 1 (PGK-1) internal control plasmid was obtained from M. Rudnicki and was generated by inserting an *Acc*I-*Pst*I fragment (nucleotides 939 to 1633 of the PGK-1 cDNA) (20) into the *Pst*I site of pSP64 (Promega). The TGF-α riboprobe was constructed by inserting the 632-bp *Nco*I fragment into the corresponding site in PSL301. The generation and characterization of both MMTV/wild-type *neu* and MMTV/TGF-α mice have been described previously (10, 18).

RNase protection assays. Total RNA was isolated from tissues by guanidinium thiocyanate extraction, followed by CsCl gradient fractionation (5). The RNA yield was determined, after resuspension in sterile H_2O, by measuring the UV A_{260}. To generate the antisense *neu* probe, the template plasmids described above were linearized with *Sma*I and then subjected to an in vitro transcription reaction with T7 RNA polymerase (19). The PGK-1 internal control probe was produced by digesting the template plasmid with *Eco*NI and transcribing the product with SP6 RNA polymerase. The TGF-α riboprobe was generated by cleavage of the template plasmid with *Xho*I, followed by in vitro transcription with the T3 RNA polymerase. The RNase protection assays were performed by hybridizing the above-mentioned probes to 20 μg of total RNA as previously described (19). The protected fragments were separated on a sodium dodecyl sulfate (SDS)–6% polyacrylamide gel and subjected to autoradiography.

Immunoprecipitation and immunoblotting. Tissue samples were ground to powder under liquid nitrogen and lysed for 20 min on ice in TNE lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml). The lysates were cleared by centrifugation at 12,000 × *g* for 10 min at 4°C. Immunoprecipitations were performed by incubating 2.0 mg of the protein lysate with either 300 ng of anti-Neu monoclonal antibody 7.16.4 (6) or 300 ng of an anti-EGFR antibody (Transduction Laboratories catalog no. E12020) for 30 min at 4°C. Following incubation with protein G-Sepharose beads (Pharmacia) on a rotating platform at 4°C for 30 min, the precipitates were washed four times with TNE. The Neu and EGFR immunoprecipitates were resuspended in SDS-gel loading buffer, and the proteins were resolved on an SDS–9% polyacrylamide gel. The proteins were transferred onto a polyvinylidene difluoride membrane (Millipore) with an immunoblot transfer apparatus (Bio-Rad). Following overnight incubation in 3% bovine serum albumin (Sigma) in Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM KCl), the membrane was probed for 2 h with antiphosphotyrosine antibodies (1:500; Upstate Biotechnology, Inc.) in bovine serum albumin in Tris-buffered saline. After being washed in Tris-buffered saline–0.05% Tween 20, the blots were incubated in 3% milk in Tris-buffered saline for 1 h. The membrane was incubated with goat anti-mouse immunoglobulin G, and the proteins were visualized by the enhanced-chemiluminescence detection system (Amersham).

Ref. 24 * For studies demonstrating the in vivo association of Neu with c-Src, tumor lysates were prepared and cleared as described previously (23). Proteins were immunoprecipitated by incubating 1.0 to 2.0 mg of total cell lysate with 2 μg of anti-c-Src antibody 7D10 (Quality Biotech) for 3 h at 4°C and subsequently washed five times with lysis buffer. The samples were resolved on an SDS–8% polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane. The membrane was probed with anti-Neu antibody AB.3 (1:1,000; Oncogene Science) as previously described (23) Ref. 24

For studies examining receptor association, tumor membranes were prepared from 0.3- to 0.6-g tissue aliquots by homogenization in a detergent-free hypotonic buffer as described previously (2). Following 45 min of ultracentrifugation at 100,000 × *g* and 4°C, pellets were solubilized for 45 min in 3-[(3-cholamidopropyl)-dimethyl-amonio]-1-propanesulfonate (CHAPS) buffer (50 mM Tris [pH 8.0], 0.7% CHAPS, 50 mM NaCl, 1 mM sodium orthovandate, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml). Nonidet P-40-insoluble material was removed by centrifugation at 14,000 × *g* for 15 min. Equivalent amounts of membrane protein were immunoprecipitated with either Neu polyclonal antiserum 21N (30) or anti-EGFR polyclonal antiserum 986 (30) and Staph A cells (Calbiochem) for 1 to 2 h. After washes, both Neu and EGFR immunoprecipitates were subjected to immunoblot analyses with the 21N polyclonal antiserum or an anti-EGFR monoclonal antibody (Transduction Laboratories). For detection, horseradish peroxidase-linked sheep anti-rabbit or anti-mouse antibodies were utilized. Direct binding assays were performed primarily as described previously (23).

Histological evaluation. Complete autopsies were performed. Tissues were fixed in 4% paraformaldehyde, sectioned at 4 μm, routinely stained with hematoxylin and eosin, and examined as indicated in the legend to Fig. 4. Whole-mount analyses were performed as previously described (11).

RESULTS

Detection of TGF-α and *neu* transcripts in mammary epithelium of transgenic mice carrying both MMTV/*neu* and MMTV/TGF-α transgenes. To determine if coexpression of TGF-α and *neu* could cooperate in mammary tumorigenesis, separate strains of MMTV/*neu* and MMTV/TGF-α were interbred to generate offspring carrying both transgenes. The MMTV/TGF-α strain is derived from line 29 and originates from a C57BL × DBA genetic background (18), whereas the MMTV/*neu* strain is derived from the N#202 founder animal and is derived from an inbred FVB genetic background (10). Because TGF-α-expressing females are unable to nurse their young, the F_1 progeny from this cross were generated by crossing MMTV/TGF-α males with MMTV/*neu* females. Females derived from this cross were segregated into breeding and virgin female groups. Analyses of the multiparous animals derived from this cross revealed that dual carriers possessing both the TGF-α and *neu* transgenes were incapable of nursing their young, like TGF-α-expressing females. In addition to the apparent lactation defect, multiparous female transgenic mice bearing the TGF-α and *neu* transgenes exhibited uniform hypertrophy of the mammary glands (22).

To assess whether the mammary epithelium derived from the various genotypes expressed the appropriate transgenes, RNAs derived from the mammary tumors derived from *neu*/TGF-α or *neu*/+ virgin transgenic mice were subjected to RNase protection analyses with riboprobes specific for *neu* (Fig. 1A) or TGF-α (Fig. 1B). To ensure equal loading of RNA, a PGK-1 antisense probe (20) was also included in the hybridization reaction mixtures. Examination of RNA samples derived from the tumors of eight MMTV/*neu* female mice revealed increased levels of *neu* transcripts. Interestingly, several of these tumor samples demonstrated evidence of altered transcripts (Fig. 1A, lanes 9, 10, and 13 to 15). Indeed, previous studies have demonstrated that these altered transcripts invariably encode in-frame deletions in the extracellular domain of Neu which result in its oncogenic activation (31). As expected, elevated *neu* transcript levels were observed in mammary tumors derived from bitransgenic animals harboring both transgenes. Significantly, the mammary tumor RNA samples obtained from seven dual transgene carriers did not exhibit evidence of altered transcripts that were observed in the *neu*-induced tumors (Fig. 1A, lanes 1 to 7).

An identical RNase protection analysis was performed on these RNA samples to assess the levels of TGF-α (Fig. 1B). These analyses revealed that TGF-α could be detected in

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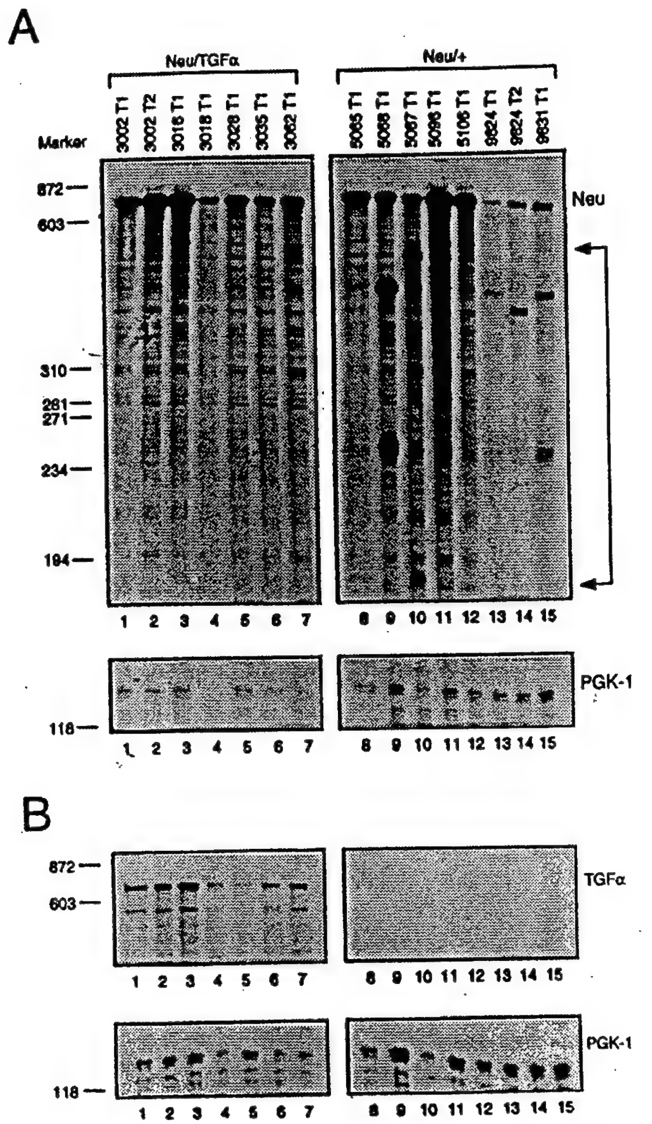

FIG. 1. Expression of Neu and TGF-α transgenes in mammary tissue of transgenic mice. (A) Neu transgene expression in mammary tissues of mice carrying the MMTV/*neu* transgene (*neu*/+) and both transgenes (Neu/TGF-α). RNA samples derived from tumor tissue (T) were subjected to RNase protection analyses. The protected wild-type *neu* transcript is 640 nucleotides long. Protected fragments corresponding to the altered *neu* transcript are indicated by arrows. Tumor RNA samples were derived from virgin female animals. An antisense riboprobe directed against the mouse PGK-1 gene was used to control for equal loading of RNA on the gel. The PGK-1 probe protects a 124-nucleotide fragment, as indicated in the lower panels. (B) The identical RNA tissue samples were hybridized with an antisense probe directed against the mouse TGF-α gene. The TGF-α antisense probe protects a 632-nucleotide fragment. The PGK-1 probe protects a 124-nucleotide fragment, as indicated in the lower panels. The numbers on the left are molecular sizes in nucleotides.

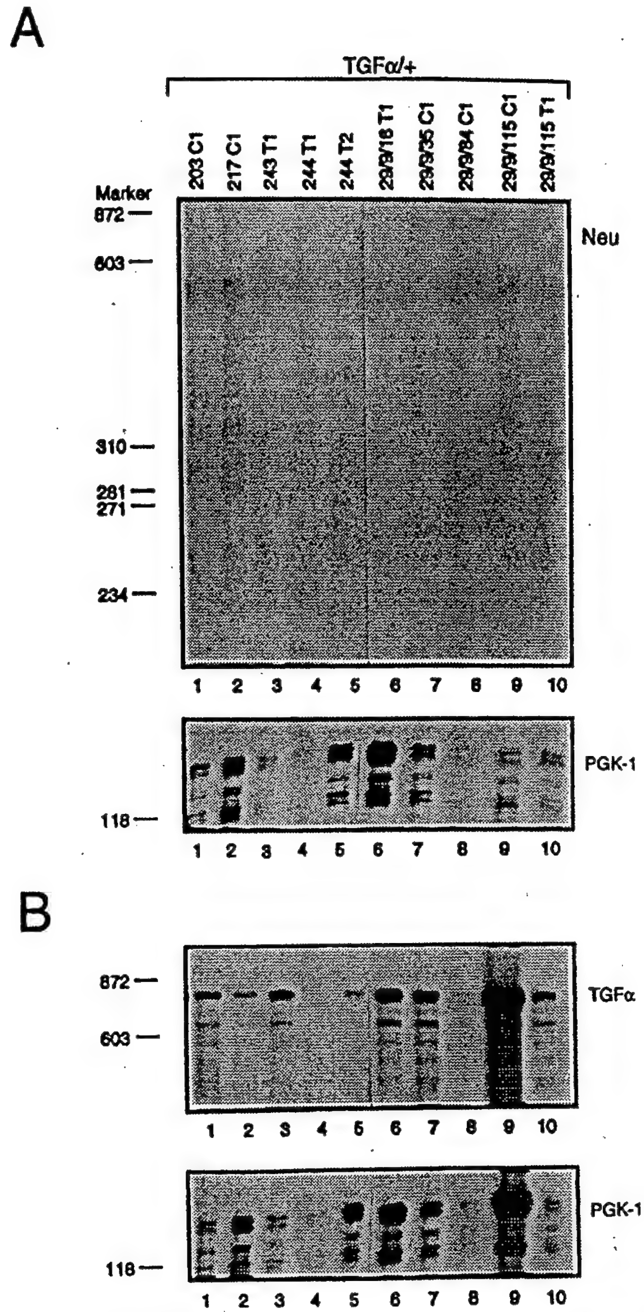

FIG. 2. Expression of Neu and TGF-α transcripts in tumors and hyperplasias derived from MMTV/TGF-α transgenic mice. (A) Endogenous Neu expression in mammary tissues of mice expressing the TGF-α transgene (TGF-α/+). Tumor (T) and cystic hyperplastic (C) tissue RNA samples from virgin female TGF-α carriers were subjected to RNase protection analyses with a *neu* riboprobe. The protected *neu* transcript is 640 nucleotides long. An antisense riboprobe directed against the mouse PGK-1 gene was used to control for equal loading of RNA on the gel. The PGK-1 probe protects a 124-nucleotide fragment, as indicated in the lower panel. (B) RNA tissue samples identical to those in panel A were probed with an antisense probe directed against the mouse TGF-α gene. The TGF-α antisense probe protects a 632-nucleotide fragment. The numbers on the left are molecular sizes in nucleotides.

mammary tumor RNA samples derived from both MMTV/TGF-α mice (Fig. 2B, lanes 1 to 10) and mice carrying both the *neu* and TGF-α transgenes (Fig. 1B, lanes 1 to 7). In contrast, no detectable transcripts corresponding to the TGF-α transgene were detected in mammary tumor RNA samples from transgenic mice carrying the *neu* transgene alone (Fig. 1B, lanes 8 to 15). Analyses of a representative sample of tumors and cystic hyperplasias derived from virgin females carrying the MMTV/TGF-α transgene alone with the identical riboprobes revealed no evidence of expression of *neu* (Fig. 2A, lanes 1 to 10); however, high TGF-α transcript levels were detected in these tissues (Fig. 2B, lanes 1 to 10). Seven MMTV/TGF-α tumor samples and five cysts were analyzed by

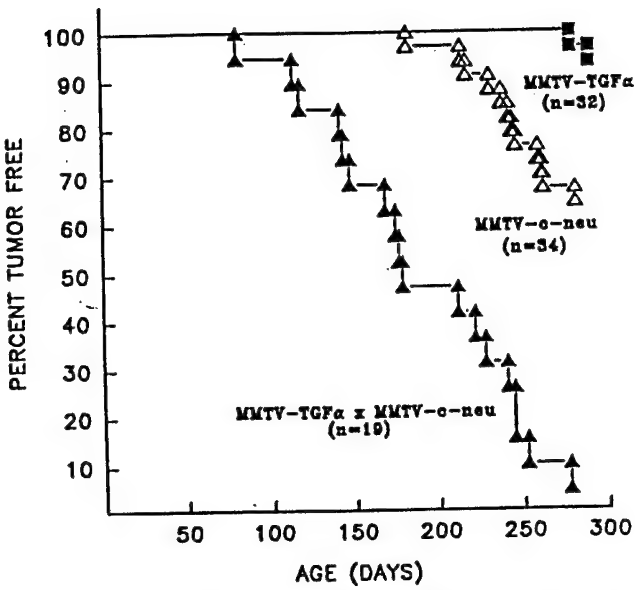

FIG. 3. Kinetics of tumor occurrence in monogenic and bigenic animals harboring the MMTV/TGF-α and MMTV/*neu* transgenes. Comparison of the kinetics of tumor formation between virgin female carriers bearing the MMTV/TGF-α, MMTV/*neu*, and both transgenes. The numbers of mice examined are indicated.

RNase protection. Taken together, these observations indicate that the dual carriers coexpress elevated levels of TGF-α and *neu* in the mammary epithelium.

Coexpression of TGF-α and *neu* in mammary epithelium results in rapid generation of multifocal mammary tumors. To test whether *neu* and TGF-α could collaborate in mammary tumorigenesis, virgin female mice carrying the *neu*, TGF-α, or both transgenes were monitored for the physical appearance of mammary tumors by palpation weekly. As shown in Fig. 3, the appearance of mammary tumors in transgenic mice expressing TGF-α or *neu* alone occurred only after long latency. Only 6% of the MMTV/TGF-α and 35% of the MMTV/*neu* mice developed palpable mammary tumors by this time (Fig. 3) and 50% of these dual carriers had tumors by 175 days, at which time neither the TGF-α- nor the *neu*-expressing mice exhibited tumors (Fig. 3). In addition to the accelerated onset of mammary tumors, the mammary tumors in the bigenic mice developed were multifocal and encompassed the entire mammary epithelium, whereas transgenic mice expressing either TGF-α or *neu* alone developed mammary tumors that were generally focal in origin.

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To explore the phenotypic differences between the various transgenic mice, mammary fat pads derived from age-matched virgin female mice were subjected to whole-mount analyses (Fig. 4). The results showed that mice carrying the *neu* transgene possessed mammary trees indistinguishable from those of virgin FVB female mice (Fig. 4B); however, the mammary trees of either the TGF-α or the *neu*/TGF-α mice were grossly abnormal (Fig. 4C and D). In fact, both the TGF-α and *neu*/TGF-α mice displayed extensive lobuloalveolar development resembling that of a normal FVB lactating female mouse (Fig. 4A). Careful examination of whole-mount preparations derived from TGF-α and *neu*/TGF-α mice revealed clear differences between the two. The alveoli present in the *neu*/TGF-α mice contained a denser cell lining in the walls (Fig. 4D) than did the large, cystically dilated alveoli found in the TGF-α mice (Fig. 4C). Consistent with these whole-mount findings, histological examination of these mammary hyperplasias derived from the MMTV/TGF-α mice had extensive lobular development (Fig. 5C) which resembled that of a lactating nontransgenic animal (Fig. 5A), except that the alveoli were irregular and dilated (Fig. 5C). Further, the interstitial stroma was edematous and had a modest increase in mononuclear and polymorphonuclear leukocytes. The mammary gland of the nulliparous female had minimal lobular development and no evidence of a stromal response (Fig. 5B). In contrast, the mammary gland of the bitransgenic animal frequently showed epithelial hyperplasia and dysplasia along with stromal inflammation (Fig. 5D). Therefore, the presence of inflammatory stroma tissue in the mammary gland is closely associated with expression of the MMTV/TGF-α transgene. The mammary tumors arising from the monogenic animals were typical of those previously reported: TGF-α-expressing animals demonstrated tubular adenocarcinomas, whereas *neu*-expressing mice typically developed nodular tumors (10, 18). The tumors arising in the bigenic *neu*/TGF-α animals were interesting in that they were either nodular (Fig. 6A) or tubular (Fig. 6B) but did not demonstrate a mixed or different morphological pattern; however, both categories of tumors coexpressed *neu* and TGF-α (Fig. 1). Together with the histological observations, these findings suggest that coexpression of *neu* and TGF-α is associated with induction of widespread morphological abnormalities in the mammary gland.

Constitutive tyrosine phosphorylation of Neu in mammary tissues coexpressing TGF-α and *neu*. The results above strongly suggest that coexpressed TGF-α and *neu* are highly synergistic in their capacity to transform the mammary epithelium. A probable biochemical explanation for this observation is that TGF-α activates Neu-associated tyrosine kinase activity by transphosphorylation through the activated EGFR, especially in light of the lack of activating mutations of *neu* in the bigenic tumors. To explore this possibility, protein extracts obtained from the mammary glands of MMTV/*neu* mice, MMTV/TGF-α mice, and dual carriers were subjected to immunoprecipitation analyses with either EGFR- or Neu-specific antiserum and immunoblotted with phosphotyrosine-specific antiserum (Fig. 7B and D, respectively). To control for the amount of protein, the same immunoprecipitates were also immunoblotted with either EGFR- or Neu-specific antiserum (Fig. 7A and C, respectively). Low but detectable quantities of EGFR protein were found in tumor samples from transgenic animals expressing either Neu or TGF-α alone (Fig. 7A, lanes 1 to 6); tumors coexpressing TGF-α and Neu expressed variable levels of EGFR (Fig. 7A, lanes 7 to 10). The levels of tyrosine-phosphorylated EGFR in these tumor samples directly correlated with the results of the immunoblot analyses (Fig. 7B). In contrast to the variable expression of EGFR, extremely high levels of tyrosine-phosphorylated Neu were detected in mammary tumors induced by the *neu* transgene alone or by both transgenes (Fig. 7D, lanes 1 to 3 and 7 to 10), which correlated with the total levels of Neu protein immunoprecipitated (Fig. 7C, lanes 1 to 3 and 7 to 10). No detectable tyrosine-phosphorylated Neu was observed in mammary protein samples from mice expressing TGF-α alone (Fig. 7C and D, lanes 4 to 6).

To further explore the mechanism by which Neu was transactivated in mammary tumors coexpressing TGF-α and *neu*, protein lysates derived from *neu*/TGF-α-, *neu*-, or TGF-α-expressing mice were subjected to reciprocal immunoprecipitation and immunoblot analyses with antisera specific to EGFR and Neu (Fig. 8). Although immunoprecipitation of these protein lysates with either Neu- or EGFR-specific antibodies, followed by immunoblot analyses with Neu-specific

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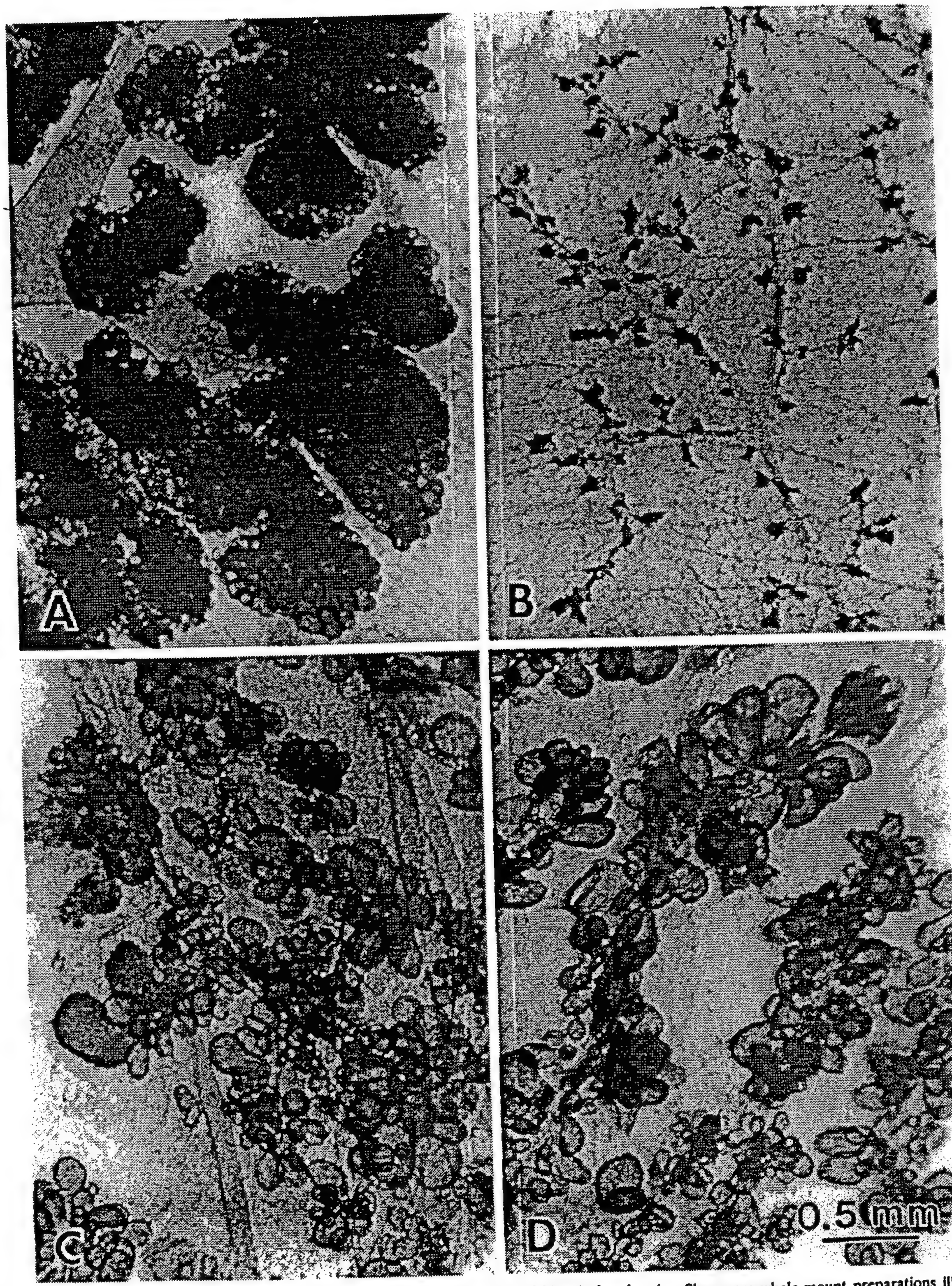

FIG. 4. Whole-mount analyses of mammary fat pads derived from monogenic and bigenic female mice. Shown are whole-mount preparations illustrating the appearance of mammary trees from a lactating FVB female (A), a virgin female with the *neu* transgene (note the numerous side buds which give the mammary tree a spiculated appearance) (B), a virgin female with the TGF-α transgene (note the well developed, cystically dilated alveoli) (C), and a virgin female with both the TGF-α and *neu* transgenes (note the larger cystic alveoli with darker walls, indicating a denser cell lining in the walls) (D). Compare these preparations with the comparable histologic preparations in Fig. 5. Magnification, ×31.5.

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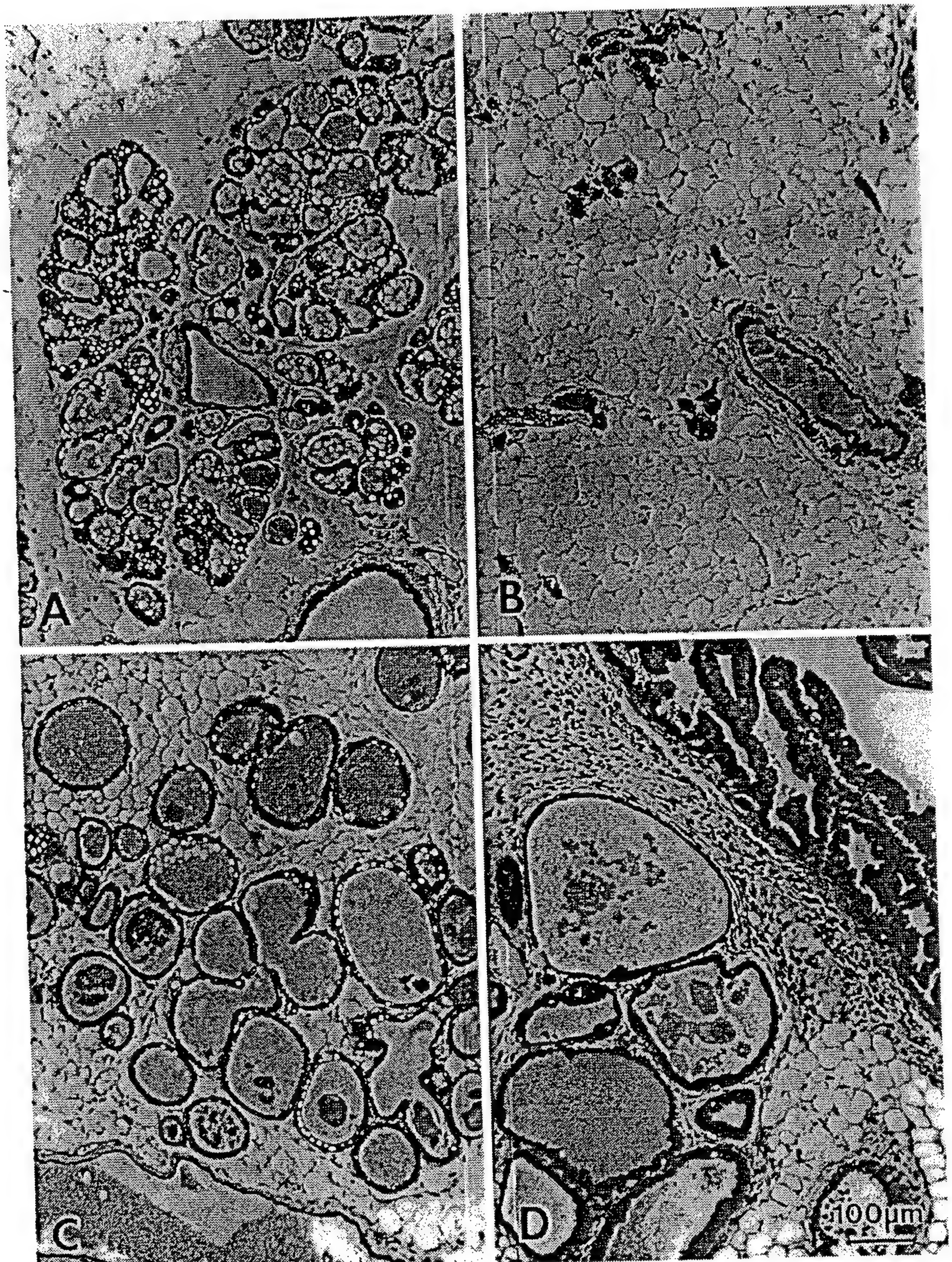

FIG. 5. Histopathology of mammary tissues derived from virgin monogenic and bigenic transgenic animals. (A) Normal FVB lactating female mouse showing lobuloalveolar development and milk production. (B) Transgenic *neu* virgin female mouse illustrating rudimentary mammary acinar development without significant luminal secretions. (C) Transgenic TGF-α virgin female mouse illustrating extensive alveolar development in comparison with a lactating mammary gland (A). Note that the alveoli are much more distended with secretory products than the FVB lactating tissue but contain fewer clear lipid vacuoles. (D) Transgenic *neu*/TGF-α virgin female mouse illustrating areas of alveolar development with papillary hyperplasia in the upper right corner. The virgin *neu*, TGF-α, and *neu*/TGF-α mice were age matched (139 days) and identical to those described in Fig. 4.

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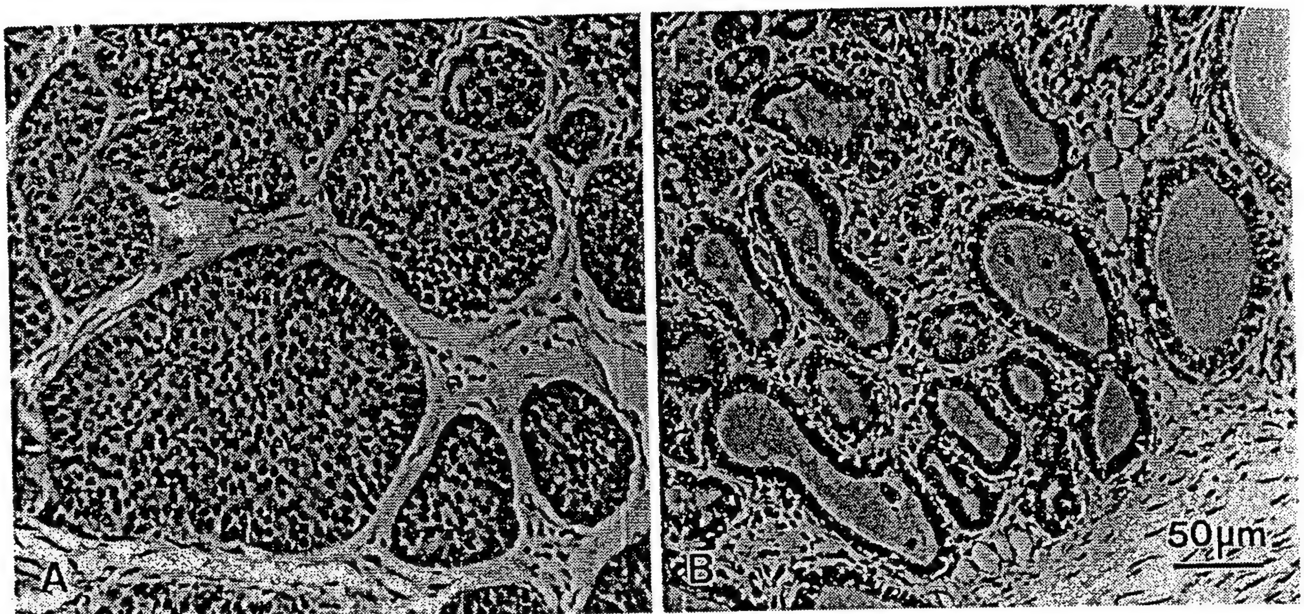

FIG. 6. Histology of the two types of mammary tumors observed in *neu*/TGF-α bigenic mice. (A) Nodular tumor typical of the Neu phenotype. (B) Tubular type typical of the TGF-α phenotype. Magnification, about ×147.

antiserum, revealed abundant Neu protein in the immunoprecipitates from the *neu*/TGF-α- and *neu*-expressing tumors, no detectable Neu was found in the EGFR immunoprecipitates (Fig. 8A). Conversely, immunoprecipitation with EGFR-specific antibodies, followed by immunoblot analyses with Neu-specific antiserum, failed to demonstrate the presence of EGFR in these complexes (Fig. 8B). Thus, under these experimental conditions, we could not detect a physical interaction between the EGFR and Neu in bigenic mice, and these findings indicate that transactivation of Neu by TGF-α does not involve the formation of stable Neu-EGFR heterodimers.

Induction of mammary tumors by *neu* and *neu*–TGF-α transgenes correlates with the capacity of Neu to associate with the c-Src tyrosine kinase in vitro and in vivo. One possible explanation for the observed synergy between Neu and TGF-α/EGFR is that these closely related type 1 receptors recruit distinct but complementary pathways. Indeed, we have previously demonstrated that activation of c-Src by the activated EGFR in fibroblasts is mediated by direct and specific association of c-Src with Neu (23). To test whether the c-Src signaling pathway was also recruited to Neu in *neu*/TGF-α-expressing tumors, Neu immunoprecipitates derived from *neu*, TGF-α, and *neu*/TGF-α tumor samples were resolved on an SDS-polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, and probed with a radiolabeled glutathione *S*-transferase (GST) fusion protein containing the SH2 domain of c-Src (GSTag–c-Src–SH2) (Fig. 9A). Consistent with previous observations (23), the radiolabeled GSTag–c-Src–SH2 probe bound to the Neu immunoprecipitates derived from mammary tumors expressing Neu alone (Fig. 9A, lanes 1 to 3). By contrast, the radiolabeled fusion protein failed to bind the Neu immunoprecipitates derived from the TGF-α-induced tumors (Fig. 9A, lanes 4 to 6). An identical analysis of the Neu immunoprecipitates derived from tumors coexpressing both Neu and TGF-α revealed that they also bound strongly to the radiolabeled GSTag–c-Src–SH2 probe (Fig. 9A, lanes 7 to 10). The observed binding of the GSTag–c-Src–SH2 probe is likely specific to tyrosine-phosphorylated Neu, since previous studies have demonstrated that the radiolabeled GSTag–c-Src–SH2 probe cannot bind comparable levels of tyrosine-phosphorylated EGFR (23). Thus, Neu derived from *neu*/TGF-α tumors is capable of interacting with c-Src in a direct manner in vitro.

To confirm that c-Src could interact with Neu in vivo, the same protein lysates were immunoprecipitated with c-Src-specific antiserum and subjected to immunoblot analyses with Neu-specific antiserum (Fig. 9B). Consistent with the in vitro binding data, Neu protein was found in c-Src immunoprecipitates derived from tumors expressing *neu* alone or coexpressing both *neu* and TGF-α (Fig. 9B, lanes 1, 2, and 5 and 7) but was absent from tumors expressing TGF-α alone since the latter fail to express detectable levels of Neu (Fig. 9B, lanes 3 and 4). Taken together, these observations suggest that transactivation of nonmutated Neu by the activated EGFR results in recruitment of the c-Src signaling pathway.

DISCUSSION

Our results show that coexpression of *neu* and TGF-α in the mammary epithelium of transgenic mice results in the induction of multiple growth disturbances in the mammary epithelium, leading to tumor formation. We also present evidence that the occurrence of these growth disturbances correlates with the constitutive activation of the tyrosine kinase activity of Neu. These observations suggest that TGF-α and *neu* cooperate in mammary tumorigenesis, possibly through transactivation of Neu by the EGFR.

The phenotype exhibited by transgenic mice coexpressing TGF-α and *neu* provides important insight into the interaction of EGFR family members in mammary tumorigenesis. Virgin female mice coexpressing TGF-α and *neu* demonstrated dramatic and distinct mammary morphological differences in comparison with either parental strain (Fig. 4 and 5). In addition, dual transgene carriers developed mammary tumors with greater penetrance and shorter latency than either *neu* or TGF-α animals alone (Fig. 3). One of the most striking features of the mammary tumor tissue derived from bigenic animals is the hyperproliferation of the stromal tissue adjacent to the neoplastic mammary epithelium. The occurrence of in-

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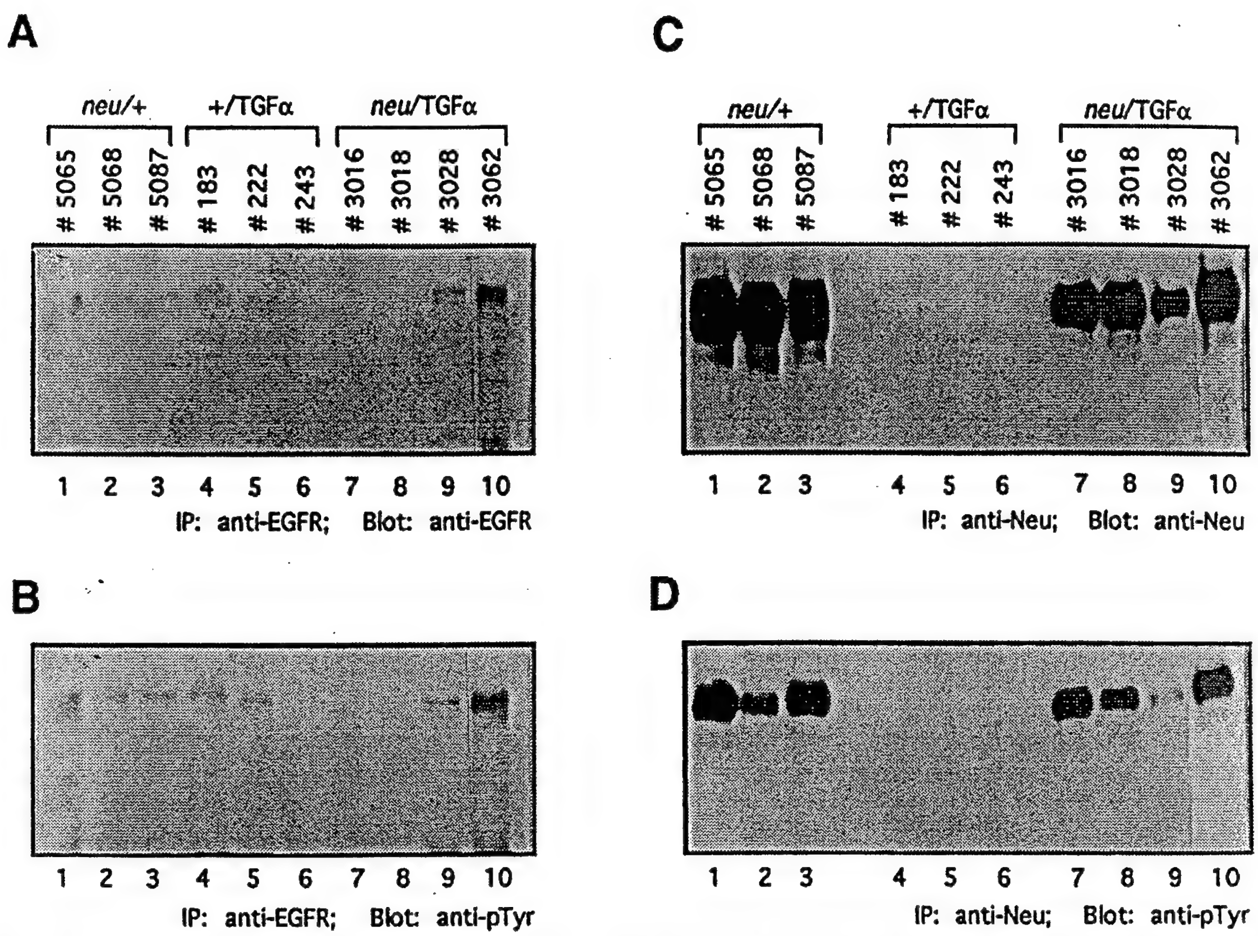

FIG. 7. Mammary tumor tissue from bigenic *neu*/TGF-α mice possess constitutively activated Neu. (A) Protein lysates from tumor tissue carrying either the MMTV/*neu* transgene (*neu*/+), the MMTV/TGF-α transgene (TGF-α/+), or both transgenes (*neu*/TGF-α) were immunoprecipitated (IP) with an anti-EGFR antibody and then subjected to immunoblot analysis with the same antiserum. (B) Tissue lysates identical to those in panel A were immunoprecipitated with anti-EGFR serum and subjected to immunoblot analyses with antiphosphotyrosine antibody 4G10. (C) Protein lysates identical to those in panel A were immunoprecipitated with the 7.16.4 monoclonal (anti-Neu) antibody and then subjected to immunoblot analysis with anti-Neu polyclonal antibody AB.3 (Oncogene Sciences). (D) Tissue lysates identical to those in panel A were immunoprecipitated with the 7.16.4 monoclonal (anti-Neu) antibody and subjected to immunoblot analysis with antiphosphotyrosine antibody 4G10.

flammatory stroma in these tumors was due to the expression of TGF-α, since the MMTV/TGF-α mice also developed inflammatory stroma adjacent to the mammary epithelial hyperplasias. Because the MMTV promoter-enhancer is normally not active in the adjacent stromal tissue (21), the stromal hyperplasias observed in these animals were likely the consequence of local paracrine stimulation of the adjacent stromal cells by the adjacent TGF-α-expressing epithelial cells. Coexpression of TGF-α and *neu* in the mammary epithelia results in the epithelial dysplasias which frequently progressed to mammary adenocarcinomas (Fig. 4, 5, and 6). These observations strongly suggest that TGF-α and Neu can cooperate during mammary tumorigenesis in vivo.

The rapid induction of mammary tumors in the dual bigenic female mice correlates with elevated expression of both the TGF-α and *neu* transgenes. Interestingly, the mammary tumors induced by *neu* alone displayed evidence of altered transcripts (Fig. 1A). Indeed, previous studies have demonstrated that these altered transcripts encode mutant Neu proteins which possess constitutive tyrosine kinase activity (31). Consistent with these data, the levels of tyrosine-phosphorylated elevated (Fig. 7D). In contrast to these observations, altered *neu* transcripts were not detected in mammary tumors coexpressing TGF-α and *neu* (Fig. 1A). Nonetheless, tyrosine-phosphorylated of nonmutated Neu was detected in these tumor tissues, thus supporting EGFR-mediated transactivation of the *neu* proto-oncogene product (Fig. 7D). In addition to tyrosine-phosphorylated Neu, various levels of tyrosine-phosphorylated EGFR were also detected in mammary tumors from transgenic mice coexpressing TGF-α and *neu* (Fig. 7B). The reason for the highly variable EGFR levels in these tumors does not appear to be sampling error, since the same samples possessed elevated Neu. It is conceivable that the different ratios of the *neu* transgene to the endogenous EGFR may influence the phenotype exhibited by the tumors arising in these dual carriers. In this regard, it is interesting that the tumors arising in these bigenic mice exhibited a tubular or nodular phenotype (Fig. 6). However, determination of whether the ratio of the *neu* transgene to the endogenous EGFR influences these phenotypes awaits further analyses.

Unlike the tumors arising in parental MMTV/*neu* mice, which frequently possess activating mutations in the transgene

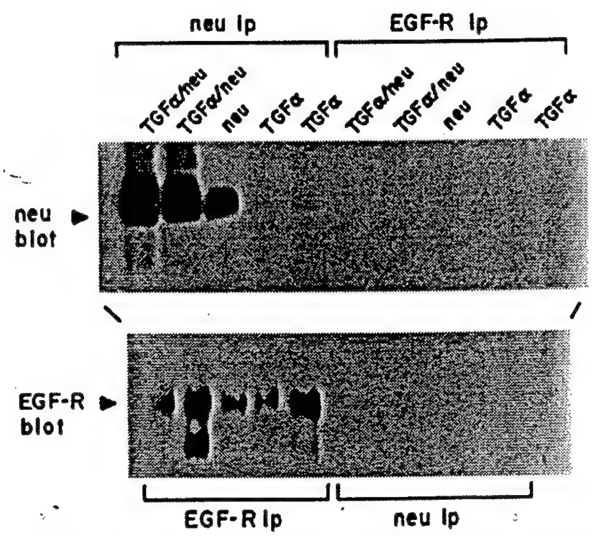

FIG. 8. Lack of detectable EGFR-Neu association in transgenic mammary tumors. Tumor membranes were prepared as described in Materials and Methods. Four hundred micrograms of membrane protein from each tumor was immunoprecipitated (ip) for 2 h with anti-Neu polyclonal antibody 21N or 986 anti-EGFR serum and Staph A cells. Precipitates were then subjected to immunoblot analysis with Neu (top panel)- or EGFR (bottom panel)-specific antiserum. Despite a detectable level of precipitable EGFRs in all of the tumors, EGFR was undetectable in all of the Neu immunoprecipitates.

mary tumorigenesis in bigenic *neu*/TGF-α mice (Fig. 1A, lanes 1 to 7). One possible explanation for the lack of activating *neu* mutations in these tumors is that Neu is activated through association with the EGFR. Consistent with this hypothesis, several groups have demonstrated in both fibroblasts and mammary epithelial cell lines that Neu can be transphosphorylated by the activated EGFR following EGF stimulation (1, 8, 13, 35). In fact, transphosphorylation of Neu can be mediated through the formation of a Neu-EGFR heterodimer (14). Moreover, EGFR-Neu heterodimers exhibit a 10-fold greater affinity for EGFR ligands (39). However, in tumors derived from *neu*/TGF-α mice, stable Neu-EGFR heterodimers were not detected (Fig. 8). Therefore, if heterodimerization between EGFR and Neu is involved in the synergistic induction of mammary tumors, the formation of these complexes is likely transient. This possibility cannot be ruled out by our experimental methods. On the other hand, these heterodimers have been reported only in cells with $>10^5$ EGF-binding sites per cell and after the addition of >10 nM exogenous EGF (8, 39). A lower level of EGFR in *neu*/TGF-α breast tumors may not allow adequate stoichiometric interactions between both RTKs and thus explain our inability to detect receptor heterodimerization. The ability of Neu to cooperate with the activated EGFR is consistent with a number of previous studies. For example, it has been demonstrated that EGFR and Neu can cooperate to transform cell lines in vitro (14). Conversely, it has been shown that administration of antibodies directed against either the EGFR or Neu reverses the transformed phenotype of cells coexpressing both Neu and the EGFR (38).

Although it is clear from these results, as well as other observations, that activation of the Neu RTK by TGF-α results

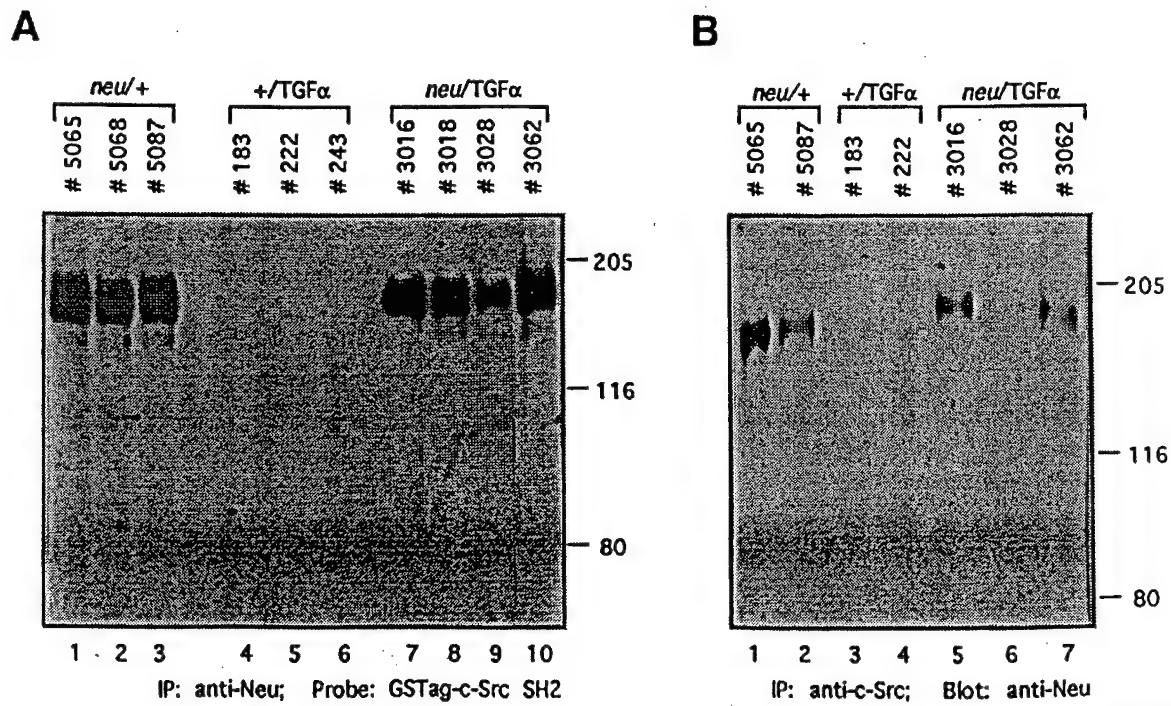

FIG. 9. c-Src is complexed with tyrosine-phosphorylated Neu in *neu*/TGF-α tumors in vitro (A) and in vivo (B). (A) Anti-Neu immunoprecipitates (Anti-Neu) from *neu*/+-, +/TGF-α-, and *neu*/TGF-α-expressing tumors were resolved in an SDS-polyacrylamide gel, blotted onto a polyvinylidene difluoride membrane, and probed with

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in synergistic transformation of mammary epithelial cells, the molecular basis for this cooperation is unclear. It is conceivable that activation of these closely related RTKs results in the recruitment of distinct but complementary signaling pathways to each of these receptors that then cooperate to transform the mammary epithelial cells. This hypothesis implies that each of * these type 1 RTKs is coupled to a distinct signaling pathway. In fact, several studies have suggested that coupling of the EGFR to the phosphatidylinositol 3′-kinase requires participation of the c-*erbB*-3 RTK (28, 34). We have demonstrated that the direct and specific interaction of c-Src with Neu is involved in signaling by the activated EGFR (23). Consistent with these earlier observations, we have shown that in mammary tumors induced by coexpression of *neu* and TGF-α, c-Src is complexed both in vitro and in vivo with tyrosine-phosphorylated Neu (Fig. 9). Although preliminary analyses suggested that c-Src activity was elevated in these *neu*- and TGF-α-coexpressing tumors, precise quantitation of the specific activity of c-Src in these tumors was problematic because of the extensive inflammatory stroma present in these tumors (Fig. 6).

Although these studies strongly suggest that TGF-α cooperates with Neu through the activated EGFR, it is unclear whether activation of EGFR is necessary for the induction of mammary tumors by Neu. However, several recent studies suggest that the activity of the EGFR is required for normal mammary epithelial proliferation. For example, a naturally occurring mouse mutant known as waved-2, which possesses a mutation in the EGFR catalytic domain that renders the EGFR functionally inactive (17), exhibits a severe lactation defect (7). Crosses between MMTV/*neu* transgenic mice and waved-2 mice should allow this question to be addressed.

ACKNOWLEDGMENTS

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Direct and specific interaction of c-Src with Neu is involved in signaling by the epidermal growth factor receptor

Senthil K Muthuswamy[1] and William J Muller[1,2]

Institute for Molecular Biology and Biotechnology, [1]Department of Biology, [2]Department of Pathology, McMaster University, Hamilton, Ontario, Canada

Transgenic mice expressing either the activated or wild type *neu* oncogene heritably develop metastatic mammary tumors. Tumor development in this transgenic mouse model correlates with activation of the Neu tyrosine kinase. Recently, we have shown that these Neu-induced mammary tumors possess elevated c-Src tyrosine kinase activity. Here, we demonstrate that c-Src requires tyrosine phosphorylated Neu for its ability to associate with Neu *in vivo* and this association is likely the result of a direct physical binding of c-Src SH2 domain to the tyrosine phosphorylated Neu. By contrast, the c-Src SH2 domain did not interact directly with tyrosine phosphorylated EGFR. Moreover, in established cell lines expressing elevated levels of EGFR, EGF stimulation results in transphosphorylation of Neu and formation of complexes between c-Src and tyrosine phosphorylated Neu. Taken together, these observations suggest that activation of c-Src by these two closely related EGFR family members results from a direct and specific interaction of c-Src with tyrosine phosphorylated Neu.

Keywords: Neu; EGFR; c-Src; SH2; receptor signaling

Introduction

Growth factors elicit their mitogenic response by activating the intrinsic protein tyrosine kinase activity of their cognate receptors. Neu (c-*erb*B-2) is a member of epidermal growth factor receptor (EGFR/c-*erb*B-1) family referred to as class I receptors (Ullrich and Schlessinger, 1990). Class I receptors share extensive sequence homology including two cysteine rich extracellular domains, a transmembrane domain, a cytoplasmic tyrosine kinase domain and a carboxyl tail. Besides *neu* and EGFR, the EGFR family include the recently identified *erb*B-3 (Kraus *et al.*, 1989; Plowman *et al.*, 1990) and *erb*B-4 (Plowman *et al.*, 1993) genes. Overexpression of the EGFR family members has been implicated in a number of human cancers. For example, amplification and consequent overexpression of *neu* has been observed in more than 20% of breast cancers (Slamon *et al.*, 1987, 1989) and is inversely correlated with the survival of the patient (Gullick *et al.*, 1990; Paterson *et al.*, 1991).

Further evidence for the role of *neu* in mammary tumorigenesis derives from observations made with transgenic mice expressing a constitutively active Neu possessing a point mutation in the transmembrane domain (Bargmann *et al.*, 1986), under the control of Mouse Mammary Tumor Virus (MMTV) promoter/enhancer (Muller *et al.*, 1988; Bouchard *et al.*, 1989). In several of these transgenic strains, high level expression of activated *neu* resulted in the development of multifocal mammary tumors that affected every female carrier (Muller *et al.*, 1988). Consistent with these studies, mice expressing the wild type *neu* transgene under the control of MMTV promoter/enhancer also develop mammary tumors (Guy *et al.*, 1992). In mice expressing wild type *neu*, the induction of mammary tumors correlates with the activation of the Neu tyrosine kinase that frequently occurs as a result of activating mutations in the transgene (Siegel *et al.*, 1994). These observations suggest that the rate limiting step in the induction of mammary tumors expressing *neu* is the activation of its tyrosine kinase activity.

Although it is clear from both these transgenic and clinical observations that activation of Neu is a key step in mammary tumorigenesis, the molecular basis for the sensitivity of mammary epithelium to the oncogenic action of *neu* is unclear. Activation of Neu tyrosine kinase results in the recruitment and functional activation of several cellular proteins with the Src homology 2 (SH2) domain. For example, cytoplasmic SH2 containing proteins such as the GTPase activating protein, Shc and PLCγ1 are phosphorylated on tyrosine residues following activation of the Neu tyrosine kinase (Fazioli *et al.*, 1991; Peles *et al.*, 1991; Segatto *et al.*, 1993; Dougall *et al.*, 1994).

EGF stimulation of cells expressing high levels of EGFR results in elevation of tyrosine kinase activity of the Src family members (c-Src, c-Yes and Fyn) (Osherov and Levitzki, 1994). Moreover fibroblasts overexpressing c-Src are hyperresponsive to EGF mediated mitogenesis (Luttrell *et al.*, 1988; Wilson *et al.*, 1989). More recently, we and others have demonstrated that physical complexes between c-Src and Neu (Muthuswamy *et al.*, 1994) and c-Src and EGFR (Luttrell *et al.*, 1994) can occur and these complexes correlate with elevated c-Src activity. These observations suggest that the c-Src tyrosine kinase may be involved in signaling by these EGFR family members.

While c-Src can complex with both EGFR and Neu, it is unclear whether c-Src interacts directly with both of these tyrosine phosphorylated receptors. Because both Neu and EGFR can heterodimerize (King *et al.*, 1988; Stern and Kamps, 1988; Kokai *et al.*, 1989; Wada *et al.*, 1990; Qian *et al.*, 1992), it is conceivable that the observed complexes of c-Src with Neu and EGFR are due its interaction with ony one of these

Correspondence: WJ Muller
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receptors. To further elucidate the mechanism by which c-Src and EGFR family members interact, we examined the ability of a radiolabeled Glutathione-S-transferase (GST)-c-Src SH2 fusion protein to directly bind denatured tyrosine phosphorylated Neu. The results revealed that the c-Src SH2 fusion protein could directly bind Neu in tyrosine phosphorylation dependent manner. Interestingly, the c-Src SH2 fusion protein was unable to interact directly with tyrosine phosphorylated EGFR. To test the hypothesis that EGFR stimulated c-Src activity was through transphosphorylation of Neu, protein extracts derived from cells stimulated with Epidermal Growth Factor (EGF) were immunoprecipitated with Neu, EGFR and Src specific antibodies and subjected to immunoblot analyses with anti-phosphotyrosine and anti-Neu antibodies. The results showed that EGF stimulation resulted in the formation of complexes between c-Src and tyrosine phosphorylated Neu. By contrast, EGF stimulation did not result in the formation of comparable complexes between EGFR and c-Src. These results suggest that activation of c-Src tyrosine kinase by these two closely related EGFR family members occurs through a direct and specific interaction of c-Src with tyrosine phosphorylated Neu.

Results

Association of c-Src with Neu is tyrosine phosphorylation dependent

Although previous studies have shown that c-Src and Neu associate with each other (Muthuswamy *et al.*, 1994) it is unclear whether this *in vivo* association requires tyrosine phosphorylated Neu. To address this question, protein extracts derived from Rat-2 cell lines expressing activated *neu* (Val 664 to Glu, Bargmann *et al.*, 1986) under the inducible MMTV promoter/enhancer (NT.12, Muthuswamy *et al.*, 1994) were subjected to immunoprecipitation and immunoblot analysis with c-Src and Neu specific antisera. To confirm that this cell line could be induced to express tyrosine phosphorylated Neu, lysates were prepared from cells grown either in the presence or absence of dexamethasone. Although dexamethasone induction of this cell line resulted in a marginal increase in the levels of total Neu (Figure 1A), the levels of tyrosine phosphorylated Neu were dramatically elevated upon stimulation with dexamethasone (Figure 1B). The elevated levels of tyrosine phosphorylated Neu can be largely attributed to the fact that the activated Neu protein expressed under the control of MMTV promoter/enhancer is 20-fold more active than the endogenous Neu (Bargmann and Weinberg, 1988). As expected, tyrosine phosphorylated Neu could not be detected in lysates derived from the Rat-2 parental cell line grown in the presence or in the absence of dexamethasone (Muthuswamy *et al.*, 1994 and unpublished observations). This suggests that the increase in the levels of tyrosine phosphorylated Neu in NT.12 cells was due the ability of dexamethasone to induce expression of the activated *neu* gene under the control of the MMTV promoter/enhancer.

To assess whether the *in vivo* association of Neu and c-Src required tyrosine phosphorylated Neu, c-Src was immunoprecipitated from both dexamethasone induced and uninduced cell lysates and subjected to immunoblot analyses with anti-Neu antibodies (Figure 1C), or with an antisera that recognizes c-Src (Figure 1D). The results revealed that c-Src complexed only with tyrosine phosphorylated Neu (Figure 1C, lane 4). The inability to detect Neu in the uninduced lysates was not due to difference in the levels of c-Src because comparable levels of c-Src were detected in both induced and uninduced extracts (Figure 1D compare lanes 3 and 4). These observations indicate that the physical association of c-Src with Neu is dependent on tyrosine phosphorylation of Neu.

To assess whether the physical association of c-Src with Neu correlated with an increase in the specific activity of c-Src, we measured the capacity of c-Src immunoprecipitates to phosphorylate acid denatured

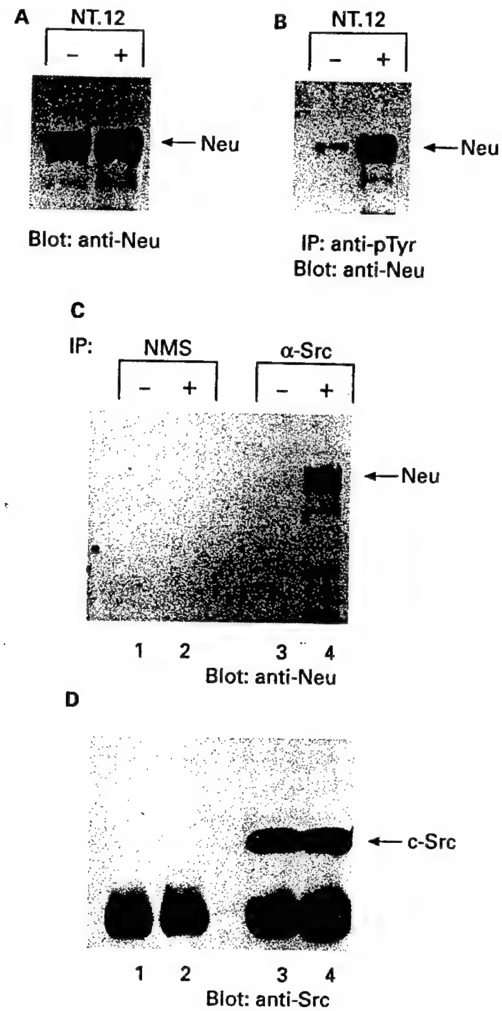

Figure 1 *In vivo* association between c-Src and Neu is dependent on the presence of tyrosine phosphorylated Neu. NT.12 cells expressing activated Neu under the control of MMTV promoter/enhancer were grown both in the presence (+) and absence (−) of dexamethasone. (**A**) Anti-Neu immunoblot of total cell lysates. (**B**) The same batch of cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and probed with anti-Neu antibodies. (**C**) c-Src was immunoprecipitated (α-Src, lanes 3 and 4) from the same batch of lysates and probed with anti-Neu antibodies. Normal mouse serum (NMS) was used as a non-specific control (lanes 1 and 2). (**D**) The blot in panel **C** was immunoblotted with an antibody that recognizes c-Src

enolase *in vitro*. The results revealed that tyrosine kinase activity of c-Src increased by 2.3-fold in dexamethasone treated NT.12 cells when compared to that observed in untreated cells (Figure 2A). The increase in the c-Src kinase activity was due to a change in the intrinsic kinase activity of c-Src since control c-Src immunoprecipitates probed with anti-Src antibodies showed identical levels of c-Src protein both in uninduced and induced conditions (Figure 2B). No difference in c-Src kinase activity was observed in the Rat-2 parental cell line grown in the presence or absence of dexamethasone (data not shown). These observations suggest that activation of c-Src occurs as a direct consequence of its ability to complex with tyrosine phosphorylated Neu.

The SH2 domain of c-Src interacts directly with Neu in a tyrosine phosphorylation dependent manner

Although these results suggest that c-Src associates with tyrosine phosphorylated Neu *in vivo*, it is unclear whether this association was a direct interaction of c-Src with Neu or occurred through the mediation of other protein(s). To determine whether the c-Src SH2 domain could directly bind to denatured Neu, total cell lysates, control immunoprecipitates and anti-Neu immunoprecipitates from a Neu expressing mammary epithelial cell line (NAFA, Muthuswamy *et al.*, 1994) were resolved on SDS–PAGE, transferred onto PVDF membranes and probed with radiolabeled GST fusion protein containing the SH2 domain of c-Src (GSTag-c-Src SH2) as a probe (Figure 3). As shown in Figure 3A, radiolabeled GSTag alone did not bind to any protein in total cell lysates or to the Neu immunoprecipitate (lanes 1–3). As a positive control, we probed an identical membrane with a radiolabeled GSTag-

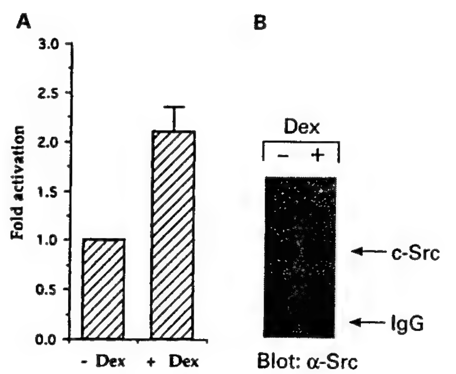

Figure 2 Increase in the specific activity of c-Src upon expression of kinase active Neu. Lysates from NT.12 cells grown both in the presence (+Dex) and in the absence (−Dex) of dexamethasone (Dex) were immunoprecipitated with Src specific antibodies and the immunoprecipitates were incubated with [γ-^{32}P]ATP and acid denatured enolase as an external substrate. The radioactivity transferred onto the enolase was quantitated by phosphorImager analyses. (**A**) The increase in c-Src kinase activity following Dex induction is shown as fold increase over the kinase activity observed in the absence of dexamethasone induction. The graph represents the average fold activation observed in four independent experiments. (**B**) A part of the immunoprecipitates used in the kinase assay (A) was immunoblotted with Src specific antisera (α-Src) and [^{125}I]anti-mouse secondary antibody

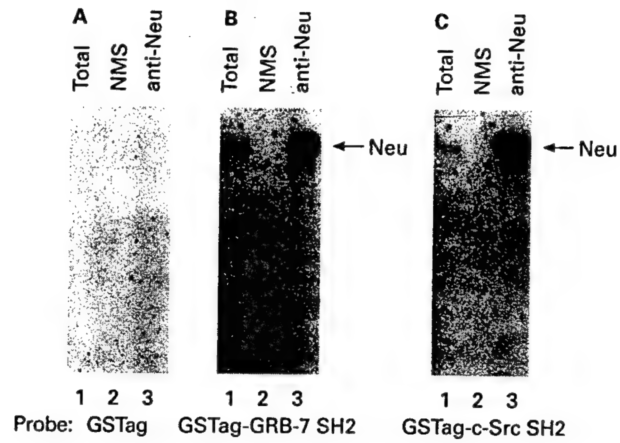

Figure 3 c-Src SH2 domain interacts directly with denatured Neu. Anti-Neu immunoprecipitates (anti-Neu) and total lysates (Total) from a mammary epithelial cell line (NAFA) were resolved in a SDS–PAGE and blotted onto a PVDF membrane. Duplicate membrane strips were probed with radiolabeled GSTag alone (**A**, negative control) or GSTag-GRB-7 SH2 (**B**, positive control) or GSTag-c-Src SH2 (**C**). Normal mouse serum (NMS) immunoprecipitate was used a non-specific control

Grb-7 SH2 fusion protein that has been previously demonstrated to directly bind Neu (Stein *et al.*, 1994a). As expected, the Grb-7 SH2 domain bound to Neu both in total extracts and in Neu immunoprecipitates (Figure 3B, lanes 1 and 3). Similar analyses using radiolabeled c-Src SH2 (GSTag-c-Src SH2) fusion protein revealed that, like Grb-7, the c-Src SH2 containing fusion protein could directly interact with denatured Neu (Figure 3C, lanes 1 and 3). These observations indicate that the complexes observed between c-Src and Neu may occur through direct interaction of c-Src SH2 domain with Neu.

To determine whether this interaction required tyrosine phosphorylated Neu, Neu was immunoprecipitated from dexamethasone induced or uninduced NT.12 cells (Figure 1A and B) using either antibodies raised against a C-terminal peptide (Ab-3) or the extracellular domain (7.16.4) of Neu (Drebin *et al.*, 1985). The immunoprecipitates were resolved on a SDS–PAGE, transferred onto PVDF membrane and probed with a radiolabeled GSTag-c-Src SH2 fusion protein. As shown in Figure 4A, the GSTag-c-Src SH2 fusion protein bound weakly to Neu immunoprecipitated from the uninduced extracts (lanes 1 and 2). By contrast, Neu immunoprecipitates derived from the induced extracts bound the GSTag-c-Src SH2 domain stronger than the immunoprecipitates from uninduced lysates (Figure 4A, compare lanes 1 and 4; lanes 2 and 5). To assess whether the strength of interaction correlated with the extent of Neu tyrosine phosphorylation the same set of immunoprecipitates were subject to immunoblot analyses with anti-phosphotyrosine antibodies (Figure 4B). The results showed that the strength of the interaction between the radiolabeled c-Src SH2 fusion protein and Neu directly correlated with the state of Neu tyrosine phosphorylation. The difference in the amount of Neu protein immunoprecipitated by Ab.3 (lane 4) and 7.16.4 (lane 5) is due to difference in the ability of the antibodies (Ab.3 and

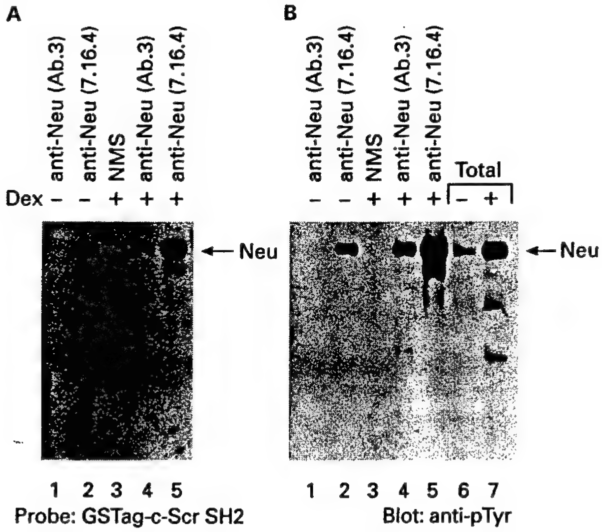

Figure 4 Direct binding of c-Src SH2 domain with denatured Neu is dependent on tyrosine phosphorylation of Neu. Equal amount of lysates from NT.12 cells grown both in the presence (+) and absence (–) of dexamethasone (Dex) were immunoprecipitated with antibodies raised against the cytoplasmic tail (Ab.3) or the extracellular domain of Neu (7.16.4). (**A**) One-half of the immunoprecipitates was resolved on a SDS–PAGE, blotted onto PVDF membrane and probed with radiolabeled GST fusion protein containing c-Src SH2 domain (GSTag-c-Src SH2). (**B**) The remaining immunoprecipitates were immunoblotted with anti-phosphotyrosine antibodies (anti-pTyr). Total cell lysates (lanes 6 and 7) were used to demonstrate the extent of dexamethasone induction and also to show the presence of low levels of tyrosine phosphorylated Neu under uninduced conditions (lane 6). Normal mouse serum (NMS) was used as non-specific control

7.16.4) to immunoprecipitate Neu. Taken together, these observations imply that c-Src SH2 domain directly binds to Neu in a tyrosine phosphorylation dependent manner.

Activation of c-Src in Neu-induced mammary tumors occurs through its interaction with Neu

The results described above suggest that activation of c-Src in Neu-induced mammary tumors correlate with its capacity to bind directly with Neu. However, it is conceivable that the other closely related EGFR family members could contribute to the elevated c-Src activity observed in these tumors. To test this possibility, we first determined whether other EGFR family members were expressed in NAFA cells and in primary Neu-induced mouse mammary tumors (Guy *et al.*, 1992). Immunoblot analyses on total cell lysates using specific antibodies to different EGFR family members (see Materials and methods) revealed that NAFA cells expressed detectable levels of *erb*B-3, while the primary Neu-induced tumors expressed detectable levels of EGFR, *erb*B-3 and *erb*B-4 (unpublished observations).

To assess whether these other family members might participate in binding to c-Src, protein extracts derived from either NAFA cells (Figure 5A) or primary Neu tumors (Figure 5C) were immunoprecipitated with antibodies directed against each of EGFR family members (EGFR, Neu, *erb*B-3, *erb*B-4) and subjected to direct binding analyses with the radiolabeled GSTag-c-Src SH2 protein. The results showed that

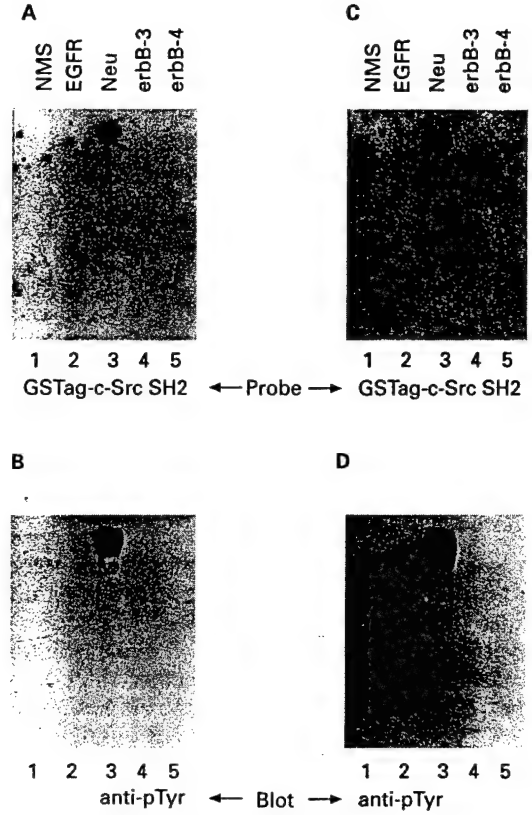

Figure 5 The c-Src SH2 domain interacts with Neu in Neu-induced mammary tumors and derived cell line. Each member of the EGFR family was immunoprecipitated using extracts from both Neu-induced mammary tumor and tumor derived epithelial cell line (NAFA). Half of the immunoprecipitates from the NAFA cells (**A**) and one half from the mammary tumor lysates (**C**) were probed with radiolabeled c-Src SH2 domain (GSTag-c-Src SH2). The remaining immunoprecipitates from NAFA cell lysates (**B**) and mammary tumor lysates (**D**) were probed with anti-phosphotyrosine antibodies (anti-pTyr). Normal mouse serum (NMS) was used as non-specific control

the c-Src SH2 probe bound only to the Neu immunoprecipitates (Figure 5A, lane 3; Figure 5C, lane 3). However, immunoblot analyses of the same immunoprecipitates with anti-phosphotyrosine antibodies indicated that Neu was the principle tyrosine phosphorylated EGFR family member (Figures 5B and 5D). After longer exposure of the blots, lower levels of tyrosine phosphorylated EGFR and *erb*B-3 were detected in the primary tumor samples (data not shown). Immunoblot analysis of EGFR, *erb*B-3 and *erb*B-4 immunoprecipitates with the same antibodies revealed that detectable amounts of the receptor proteins were immunoprecipitated (data not shown). Nonetheless, no detectable binding of c-Src to these immunoprecipitates was observed. These analyses suggest that among the EGFR family members Neu is the principle tyrosine phosphorylated receptor that c-Src SH2 domain interacts within these mammary tumors and tumor derived cell lines.

The c-Src SH2 domain does not associate directly with activated EGFR in vitro

The experiments described above indicate that in mammary tumor cells the association between c-Src

and Neu is likely responsible for the elevated Src activity observed in these tumors (Muthuswamy *et al.*, 1994). However, it is unclear whether c-Src SH2 domain could associate directly with the other EGFR family members such as EGFR that is known to induce kinase activity of the Src family members (Osherov and Levitzki, 1994). Stimulation of fibroblasts overexpressing EGFR with EGF for 1.0 min results in a threefold activation of the Src family of kinases using an antibody that recognizes c-Src, c-Yes and Fyn (Osherov and Levitzki, 1994). Similarly, we have observed a weak but consistent 1.4-fold activation of c-Src using c-Src specific antibodies (Ab327) following acute stimulation of Rat-1 fibroblasts overexpressing human EGFR (R1/hER, Wasilenko *et al.*, 1991) (unpublished observations). To assess whether the increase in c-Src activity correlated with the capacity of c-Src to interact directly with the activated EGFR, we tested the ability of c-Src SH2 fusion protein to interact with EGFR immunoprecipitates derived from either R1/hER or A431 cells after EGF stimulation. As shown in Figure 6A, we were unable to detect direct binding of c-Src SH2 domain to EGFR immunoprecipitates following EGF stimulation (lanes 2 and 4). By contrast, comparable analyses with Neu immunoprecipitates from the NAFA cell line demonstrated direct binding of the Src SH2 fusion protein to Neu (Figure 6A, lane 3). The inability to detect binding of c-Src SH2 to the EGFR was not due to lack of tyrosine phosphorylation of EGFR because immunoblot analyses of the immunoprecipitates with anti-phosphotyrosine antibodies clearly showed the presence of significant levels of tyrosine phosphorylated EGFR (Figure 6B, lanes 2 and 4). To confirm that the lack of association between EGFR and c-Src SH2 was not due difference in the levels of tyrosine phosphorylated Neu and EGFR we performed the analysis using comparable levels of tyrosine phosphorylated Neu and EGFR. The results clearly showed that under conditions where equivalent levels of tyrosine phosphorylated receptors were present we were unable to detect binding of c-Src SH2 domain to EGFR while the c-Src SH2 domain bound to Neu (data not shown).

To confirm that the tyrosine phosphorylated EGFR in the immunoprecipitates retain its ability to associate with a SH2 domain that is known to associate with EGFR. We assessed the ability of the adapter protein Grb2, which is known to associate with both phosphorylated EGFR and Neu (Egan *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Batzer *et al.*, 1994; Janes *et al.*, 1994), to associate with EGFR. As shown in Figure 7A (lanes 2–4), stimulation of R1/hER cells with EGF resulted in a marked tyrosine phosphorylation of EGFR. The same immunoprecipitates were electrophoresed through a SDS–PAGE gel, blotted onto PVDF membranes and, probed with either radiolabeled GSTag-Grb2 SH2 (Figure 7B) or GSTag-c-Src SH2 (Figure 7C) fusion proteins. The results clearly show that Grb2 SH2 domain binds directly to EGFR (Figure 7B, lanes 2–4) suggesting that the EGFR molecules in the immunoprecipitates retain their ability to associate with SH2 domains. However, as observed previously we were unable to detect association between the Src SH2 domain and the tyrosine phosphorylated EGFR (Figure 7C). In contrast control Neu immunoprecipitates bound both to Grb2 SH2 and c-Src SH2 domains (Figure 7B and C, lane 1). These observations strongly suggest that activation of c-Src by EGF may occur through a mechanism that does not require direct binding to the activated EGFR.

Figure 6 The c-Src SH2 domain does not interact directly with tyrosine phosphorylated EGFR. Lysates from EGF treated A431 epithelial cells (lane 2) or R1/hER fibroblasts (lane 4) were immunoprecipitated with anti-EGFR antibodies (anti-EGFR) and Neu was immunoprecipitated from NAFA cell lysates (anti-Neu). (**A**) One half of the immunoprecipitates was resolved on a SDS–PAGE and probed with radiolabeled c-Src SH2 domain (GSTag-c-Src SH2). (**B**) The remainder of the immunoprecipitate was immunoblotted with anti-phosphotyrosine antibody (anti-pTyr). Normal mouse serum (NMS, lane 1) was used as a non-specific control. (IP: Immunoprecipitation). The molecular weight markers are in kDa

EGF treatment of EGFR overexpressing cells results in association of c-Src with tyrosine phosphorylated Neu

Given that c-Src can bind directly to Neu and that Neu can heterodimerize with EGFR, one possible explanation for these observations is that EGF mediated activation of c-Src is occurring through a EGFR/Neu

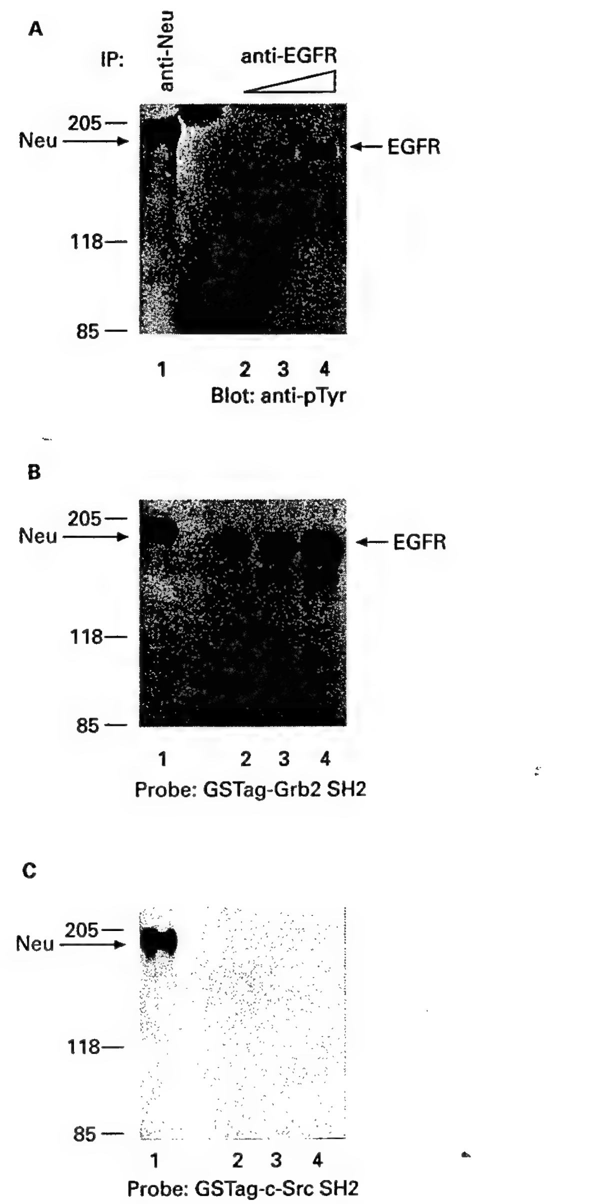

Figure 7 Tyrosine phosphorylated EGFR can bind directly to Grb2 SH2 domain but not to c-Src SH2 domain. Increasing amounts of lysates from EGF treated R1/hER cells were immunoprecipitated with EGFR-specific (anti-EGFR) antibodies (lanes 2–4). The immunoprecipitates were equally divided, resolved on a SDS–PAGE and were immunoblotted with anti-pTyr antibodies (**A**), or probed with radiolabeled Grb2 SH2 domain (GSTag-Grb2 SH2) (**B**), or probed with radiolabeled c-Src SH2 domain (GSTag-c-Src SH2) (**C**). Lane 1 in **A**, **B**, and **C** correspond to Neu immunoprecipitate from NAFA cell lysate (anti-Neu). The molecular weight markers are in kDa

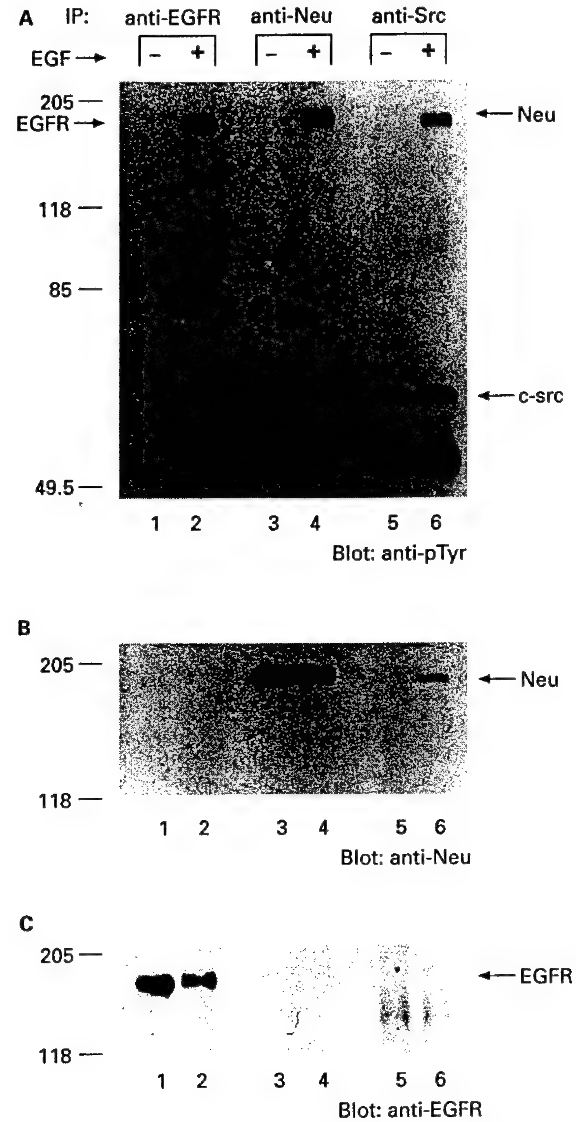

Figure 8 EGF treatment results in specific association of c-Src with Neu. Lysates were derived from R1/hER both before (−) and after (+) one minute induction with EGF. EGFR (anti-EGFR) or Neu (anti-Neu) or c-Src (anti-Src) were immunoprecipitated from the lysates and resolved on a SDS–PAGE gel. The immunoprecipitates were probed with anti-phosphotyrosine (**A**) or anti-Neu (**B**) or with anti-EGFR (**C**) antibodies. The autoradiograph in panel C was exposed almost 10 times longer than that in **B**. (IP: Immunoprecipitation). The molecular weight markers are in kDa

heterodimer. To explore this hypothesis, we examined whether EGF treatment could induce the formation of Neu/Src complexes through tyrosine phosphorylation of the endogenous Neu present in R1/hER cells. To accomplish this, cell lysates derived from either unstimulated or EGF stimulated R1/hER cells were immunoprecipitated with EGFR, Neu or Src specific antisera and subjected to immunoblot analyses with anti-phosphotyrosine antibodies (Figure 8A). The results showed that EGF administration resulted in the rapid tyrosine phosphorylation of both the EGFR (Figure 8A, lanes 1 and 2) and Neu (lanes 3 and 4). Because EGF cannot directly bind Neu (King *et al.*, 1988; Stern and Kamps, 1988), the observed tyrosine phosphorylation of Neu is likely the result of transphosphorylation of Neu by the activated EGFR. Significantly, EGF stimulation resulted in the formation of a complex between c-Src and a 185 kDa tyrosine phosphorylated protein that comigrated with Neu (Figure 8A, compare lanes 4 and 6). By contrast, we were unable to detect any tyrosine phosphorylated protein that comigrated with the EGFR in these c-Src immunoprecipitates.

To confirm that the tyrosine phosphorylated protein was Neu and not EGFR, identical sets of immunoprecipitates were immunoblotted with either a Neu (Figure

8B) or EGFR specific antibody (Figure 8C). The results confirmed that EGF stimulation of these cells resulted in the formation of specific complexes between c-Src and Neu (Figure 8B, lane 6). However, no comparable EGFR/c-Src complex was detected in these EGF stimulated cells (Figure 8C, lane 6). Taken together, these observations strongly suggest that activated EGFR stimulates c-Src through Neu.

Discussion

Our results show that activation of c-Src by two EGFR family members, EGFR and Neu occurs through specific and direct interaction of c-Src with tyrosine phosphorylated Neu. We also present evidence suggesting that EGF stimulation results in association of c-Src with tyrosine phosphorylated Neu. Taken together, these observations suggest that Neu plays a central role in the activation of c-Src by these two closely related EGFR family members.

The expression of kinase active Neu in rat fibroblasts results in a reproducible 2.3 fold increase in the specific activity of c-Src (Figure 2). Although this is a relatively modest increase as compared to the 6.7-fold activation observed in *neu*-induced mammary tumors (Muthuswamy *et al.*, 1994), this increase in c-Src kinase activity is consistent with that observed for platelet derived growth factor receptor (PDGFR) and colony stimulating factor-1 receptor (CSF-1R) activation in fibroblast cell lines (Ralston and Bishop, 1985; Kypta *et al.*, 1990; Courtneidge *et al.*, 1993). The increase in specific activity of c-Src correlated with the ability of c-Src to associate with Neu in tyrosine phosphorylation dependent manner (Figure 1). These observations demonstrate a functional link between activation of the Neu receptor tyrosine kinase (RTK) and the increase in c-Src tyrosine kinase activity and suggests that c-Src plays role in Neu-mediated signal transduction.

The activation of c-Src by Neu in Neu-induced mammary tumors and mammary epithelial cell line appear to occur through a direct interaction of c-Src SH2 domain with Neu in a tyrosine phosphorylation dependent manner (Figures 3, 4 and 5). It is possible that the direct interaction between c-Src and tyrosine phosphorylated Neu would render c-Src into a catalyticaly active conformation. However, formal proof for this hypothesis awaits future experimentation. Although immunoblot analyses indicated that other members of the EGFR family were expressed in these mammary tumors and derived cell line (unpublished observations), further biochemical analyses revealed that these members are only weakly tyrosine phosphorylated. Therefore, it is likely the elevated c-Src activity observed in the Neu-induced mammary tumors and tumor derived cell line does not involve the participation of other members of the EGFR family.

While activation of EGFR does not appear to be involved in the activation of c-Src in these mammary epithelial cells, there is considerable evidence to suggest that activation of c-Src is involved in EGF mediated mitogenesis in other cell types. For example, EGF treatment of PC12 cells or mouse fibroblasts overexpressing EGFR activates Src family of kinases (Osherov and Levitzki, 1994). Cells overexpressing c-Src show hyperresponsiveness to EGF mediated growth stimulation (Luttrell *et al.*, 1988, Wilson *et al.*, 1989) and microinjection of antibodies that recognize c-Src, c-Yes and Fyn results in a strong inhibition of EGF-induced S phase entry of fibroblasts (Roche *et al.*, 1995). Although these studies suggest that activation of c-Src tyrosine kinase plays an important step in EGF-induced mitogenesis, our results strongly suggest that c-Src does not directly interact with the activated EGFR (Figures 6, 7 and 8). Rather, our observations suggest that activation of c-Src by the activated EGFR occurs through transphosphorylation of Neu (Figure 7). Consistent with our observations, under conditions where EGF activates Src family kinases, physical association between EGFR and Src family was not detected (Osherov and Levitzki, 1994). By contrast to these observations, the formation of an activated EGFR and c-Src complex *in vivo* has been reported in MDA-MB-468 mammary tumor cells (Luttrell *et al.*, 1994). However, whether this interaction involves Neu is unclear.

Based on peptide binding approaches (Songyang *et al.*, 1993) none of the known tyrosine autophosphorylation sites on Neu correspond to the predicted c-Src consensus binding site (Hazan *et al.*, 1990, Carraway and Cantley, 1994). However, the recently identified c-Src binding site on PDGFR (DGHEY*IY*VDP) (Mori *et al.*, 1993) also does not conform to the predicted consensus sequence. In another study, a tyrosine phosphorylated peptide corresponding to tyrosine 992 in the EGFR was a more effective Src binding peptide than phosphorylated tyrosine 527 in the c-Src carboxyl tail (Luttrell *et al.*, 1994). Interestingly, among the mapped autophosphorylation sites on the EGFR Tyr 992 is the most weakly phosphorylated site (Walton *et al.*, 1990) whereas the corresponding tyrosine in *erb*B-2/Neu, tyrosine 1023, is a strong autophosphorylation site (Hazan *et al.*, 1990). It is conceivable that the differences in the ability of c-Src to bind EGFR and Neu reflects differential tyrosine phosphorylation of this site in these closely related RTKs. However, whether this conserved site on Neu is responsible for binding of c-Src awaits further analyses.

The observation that activation of c-Src in both EGFR and *neu* expressing cells occurs through tyrosine phosphorylated Neu has important implications in understanding the biological properties of these two closely related EGFR family members. For example, several studies have shown that under comparable conditions of expression and enzymatic activity, Neu is 100-fold more potent than EGFR in stimulating mitogenesis in NIH3T3 fibroblasts (Di Fiore *et al.*, 1987, 1990). More recently, it was shown that the c-Src substrate paxillin is tyrosine phosphorylated in cells expressing the cytoplasmic domain of Neu in a EGFR-*erb*B-2 chimera. Interestingly in cells expressing EGFR, tyrosine phosphorylated paxillin was not detected (Romano *et al.*, 1994). These observations suggest that the differences in the mitogenic properties of Neu and EGFR could in part be explained by the inability of the latter receptor to couple with the Src pathway.

The notion that each of these EGFR family members has distinct signaling specificity is further supported by several recent studies. For example, it

was shown that activation of the phosphatidylinositol-3′ (PI-3′) kinase by EGF, is mediated through the interaction of PI-3′ kinase with erbB-3 in a EGFR/*erb*B-3 heterodimer (Soltoff *et al.*, 1994). Consistent with these observations the 85 kDa subunit of PI-3′ kinase associates with an EGFR-*erb*B-3 chimera upon EGF stimulation (Fedi *et al.*, 1994; Prigent and Gullick, 1994). These observations suggest that activation of these closely related RTKs results in the recruitment of distinct but complementary signaling pathways. Indeed coexpression of the EGFR and Neu can act synergisticaly to transform rodent fibroblasts (Kokai *et al.*, 1989). Interestingly, a modified breast cancer cell line that does not express *erb*B-2 at cell surface, but expresses other erbB family of receptors, is defective in EGF and Neu differentiation mediated factor (NDF) mediated signaling (Graus-Porta *et al.*, 1995). This cell line shows significant reduction in its ability to facilitate activation of mitogen-activated protein kinase and to promote induction of c-*fos* gene expression in response to EGF or NDF stimulation, when compared to the parental cell line that expresses all *erb*B family receptors including *erb*B-2 on the cell surface (Graus-Porta *et al.*, 1995). This observation taken together with results presented in this report suggest that c-*erb*B-2/Neu plays a central role in signaling by the *erb*B family of receptor tyrosine kinases.

We have recently shown that c-Yes but not Fyn tyrosine kinase activity is also elevated in Neu-induced mouse mammary tumors (Muthuswamy and Muller unpublished observations). The elevated c-Src and c-Yes activity observed in these Neu-induced mammary tumors suggest these c-Src family kinases may play a role in mammary tumorigenesis. Consistent with this view, we have recently demonstrated that a functional c-Src is required for the induction of mammary tumors by PyV middle T antigen (Guy *et al.*, 1994; Muthuswamy and Muller, 1994). In addition, it has recently been demonstrated that activation of Src family of kinases are necessary for PDGFR and EGF mediated mitogenesis in fibroblasts (Twamley-Stein *et al.*, 1993; Roche *et al.*, 1995). However, it is unclear whether the function of c-Src and c-Yes are required for Neu-mediated mammary tumorigenesis. Future crosses between the MMTV/*neu* transgenic mice and either the c-*src* or the c-*yes* deficient mice (Soriano *et al.*, 1991; Stein *et al.*, 1994b) should allow this question to be addressed.

Materials and methods

DNA constructs and cell lines

The GSTag SH2 fusion for c-Src was constructed by subcloning a BamHI/EcoRI fragment containing the SH2 domain for GST-c-Src SH2 (Muthuswamy *et al.*, 1994) (amino acids 141 to 266) into a BamHI/EcoRI site in pGSTag (Ron and Dressler, 1992). pGSTag-GRB7 SH2 was a generous gift of Ben Margolis. Mouse mammary epithelial cell line NAFA was derived from an activated *neu*-induced mouse mammary tumor (Muller *et al.*, 1988). NT.12 cells expressing activated *neu* have been described earlier (Muthuswamy *et al.*, 1994). To induce the expression of activated *neu*, the NT.12 cells were grown in the presence of dexamethasone (final concentration 10^{-6} M) for a period of 24 to 36 h. Cell lines over-expressing human EGFR, R1/hER (Wasilenko *et al.*, 1991, kindly provided by Michael Weber) and A431 cells (ATCC) were induced with EGF (100 ng per ml) for 1 min, prior to lysis. All cell lines were grown in 100 mm plates to confluency ($4-6\times10^6$ cells) in modified DMEM containing 10% fetal bovine serum prior to lysis.

Immunoprecipitation and in vitro *kinase assays*

The NAFA and NT.12 cells were lysed in 0.7% 3-{(cholamidopropyl)-dimethyl-ammonio}-1-propanesulphonate (CHAPS) lysis buffer (50 mM Tris HCl 8.0, 0.7% CHAPS, 50 mM NaCl, 2 mM NaF, 1 mM sodium orthovanadate, 10 μg of leupeptin per ml, 10 μg of aprotenin per ml) as previously described (Muthuswamy *et al.*, 1994). Lysates from mouse mammary tumors were prepared as described (Muthuswamy *et al.*, 1994). The lysates were cleared by centrifugation at 4°C for 20 min. Associated proteins were immunoprecipitated by incubating 1.0–2.0 mg of total cell lysate with 2–3 μg of anti-c-Src antibody (7D10, Quality Biotech) for 3.0 h at 4°C and subsequently washed 5× with lysis buffer. Immunoprecipitation of various EGFR family members was performed by incubating lysates with anti-EGFR (Transduction labs), anti-Neu (7.16.4) or Ab-3 (Oncogene Science), anti-*erb*B-3 (RTJ.2 and C-17) and anti-*erb*B-4 (C-18) (Santa Cruz Biotechnology Inc.). The immunoprecipitates were resuspended in 1× SDS-gel loading buffer. *In vitro* kinase assays were performed as described (Muthuswamy *et al.*, 1994). Anti-Src antibody (Ab327) used in the kinase assays was kindly provided by Joan Brugge.

Immunoblotting

The samples were resolved on a SDS-8 or 9% polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane (PVDF) (Millipore). The membranes were probed with anti-Src (1:1000; Ab327), anti-Neu (Ab.3; 1:1000; Oncogene Science), anti-Yes antibodies that cross reacts with both Src and Yes (1:500; generous gift of Marius Sudol), anti-phosphotyrosine (1:750; 4G10, Upstate Biotechnology, Inc.), anti-EGFR (1:750, Transduction Labs), anti-*erb*B-3 (1:500, C-17, Santa Cruz Biotechnology Inc.) or anti-*erb*B-4 (1:500, C-18, Santa Cruz Biotechnology Inc.) antibodies as previously described (Muthuswamy *et al.*, 1994).

Direct binding assay

These assays were performed primarily as described elsewhere (Ron and Dressler, 1992; Stein *et al.*, 1994a) with minor modifications. The GSTag fusions were labeled *in vitro* as follows: 10 μl of beads harboring 15–20 μg of fusion protein were washed once with Phosphorylation buffer (DK) (50 mM Potassium phosphate, pH 7.15; 10 mM $MgCl_2$; 5 mM NaF; 4.5 mM DTT) and resuspended in 60 μl of reaction mixture containing 500 μCi of [γ-^{32}P]ATP (6000 Ci per mM, Dupont NEN) in DK buffer. The labeling reaction was started by adding 0.2 U per μl of Protein Kinase A (Sigma) and incubated at 30°C for 30 min. The unincorporated nucleotides were removed by washing 4–5 times with 1× Phosphate Buffered Saline supplemented with 5 mM NaF (1× PBS: 140 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4). Immunoprecipitates and total cell lysates were resolved on a SDS–PAGE and transferred onto PVDF membranes. The membranes were incubated at room temperature for 3.0 h in blocking buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 0.02% sodium azide, 5 mM DTT, 5% Skim milk). The membranes were subsequently probed for 2 h at room temperature in blocking buffer containing 1×10^6 c.p.m. per ml of probe and washed 4–5 times in Tris buffered saline (TBS), 0.05% Tween-20 (TBS; 20 mM Tris-HCl{pH 8.0}, 150 mM NaCl, 5 mM KCl).

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Activation of Src family kinases in Neu-induced mammary tumors correlates with their association with distinct sets of tyrosine phosphorylated proteins *in vivo*

Senthil K. Muthuswamy[1] and William J Muller[1,2]

[1]Department of Biology and [2]Department of Pathology, Institute for Molecular Biology and Biotechnology, Cancer Research Group, McMaster University, Hamilton, Ontario, Canada

Overexpression and amplification of the *erbB-2* (*neu*) is thought to play a major role in mammary cancer. Although studies suggest that Neu is directly involved in the genesis of mammary tumors, the molecular mechanism by which Neu induces tumors is not well understood. Recently, we have demonstrated that the activity of c-Src tyrosine kinase is elevated in Neu-induced mammary tumors and this elevated activity correlates with its capacity to physically associate with Neu. To explore whether other members of the c-Src family are activated in these mammary tumors, we measured the *in vitro* kinase activity of the c-Yes and Fyn kinases in protein extracts derived from mammary tumor tissue and morphological normal adjacent tissue. These analyses revealed that c-Yes kinase activity was elevated in Neu-induced tumors by comparison to the adjacent tissue. By contrast, no significant activation of the Fyn kinase was noted in these tumors. Activation of c-Yes tyrosine kinase correlated with the capacity of c-Yes to associate with Neu *in vivo* in lysates derived from primary tumor samples. Studies with Rat.2 fibroblasts overexpressing activated Neu revealed that c-Src requires the presence of tyrosine phosphoryated Neu for its ability to interact with Neu *in vivo*. Moreover, analyses using radiolabeled c-Yes SH2 fusion protein revealed that this interaction is likely occurring in a direct fashion. Although both c-Src and c-Yes kinase associate with Neu *in vivo*, a tyrosine phosphorylated protein of 89 kd (p89) was found associated with c-Src but not with c-Yes in cell lysates derived from mammary epithelial cells transformed by either Neu or PyV middle T antigen. Furthermore, this tyrosine phosphorylated protein was not detected in c-Src complexes derived from fibroblasts transformed by either Neu or PyV middle T. These observations suggest that p89 associates with c-Src only in mammary epithelial cells and not in fibroblasts.

Keywords: C-Yes; FyN; tyrosine phosphorylation; mammary tumors

Introduction

The c-Src, c-Yes and Fyn proteins are members of the Src non-receptor protein tyrosine kinase family that are expressed ubiquitously and are thought to be involved in signal transduction pathways (Erpel and Courtneidge, 1995). The Src family members possess conserved motifs termed the Src homology (SH) 2 and 3 domains that play important roles in mediating protein-protein interactions (Pawson and Schlessinger, 1993; Pawson, 1995). For example, the tyrosine kinase activity of c-Src, c-Yes and Fyn tyrosine kinases are activated by receptor tyrosine kinases (RTKs) such as the Platelet derived growth factor receptor (PDGFR) (Kypta *et al.*, 1990) and the Colony stimulating growth factor-1 receptor (CSF-lR) (Courtneidge *et al.*, 1993). c-Src is also known to associate with the Fibroblast growth factor receptor (Zhan *et al.*, 1994). The association between Src family members and these RTKs is thought to be mediated by the SH2 domains of the Src family and one or more autophosphorylated tyrosines in the cytoplasmic domain of the receptor tyrosine kinases (Twamley *et al.*, 1992; Courtneidge *et al.*, 1993; Zhan *et al.*, 1994). Recent evidence suggests that the tyrosine residues at position 579 and 581 in the PDGFR are responsible for the interaction between PDGFR and Src family members (Mori *et al.*, 1993).

Recently, we and others have demonstrated that c-Src tyrosine kinase is involved in signaling by both the Epidermal growth factor receptor (EGFR) and the Neu RTK (Luttrell *et al.*, 1994; Muthuswamy *et al.*, 1994; Osherov and Levitzki, 1994; Muthuswamy and Muller, 1995). These two receptors are members of the class 1 type of RTK that include EGFR [erbB-1], Neu [erbB-2], erbB-3, and erbB4 (Ullrich and Schlessinger, 1990; CarrawayIII and Cantley, 1994). Overexpression of the erbB family is known to play a significant role in human breast cancer (Rajkumar and Gullick, 1994). For example, amplification and overexpression of erbB-2 is known to occur in more than 20% of the breast cancer patients and has been shown to be inversely correlated with clinical prognosis (Slamon *et al.*, 1987, 1989; Gullick *et al.*, 1991; Paterson *et al.*, 1991). Consistent with these observations, overexpression of a constitutively active form of *neu* (Bargmann *et al.*, 1986) under the control of the Mouse mammary tumor virus (MMTV) promoter/enhancer in the mammary epithelial cells of transgenic mice results in the rapid development of mammary tumors (Muller *et al.*, 1988). Overexpression of wild-type *neu* under the control of the MMTV promoter/enhancer in transgenic mice also results in the development of mammary tumors albeit with long latency (Guy *et al.*, 1992b). The majority of mammary tumors in wild-type Neu expressing transgenic mice show presence of activating mutations in the extracellular domain of Neu protein encoded by the transgene (Siegel *et al.*, 1994). Although these observations suggest that the mammary epithelium is sensitive to the activation of the Neu RTK, the actual mechanism by which Neu induces mammary tumorigenesis is not yet understood.

Correspondence: WJ Muller
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A number of intracellular signaling molecules such as Phospholipase C-γ1 (Fazioli *et al.*, 1991; Peles *et al.*, 1991; Segatto *et al.*, 1992; Jallal *et al.*, 1992), GTPase activating protein (GAP) (Fazioli *et al.*, 1991; Jallal *et al.*, 1992), Shc (Segatto *et al.*, 1993), Grb2 (Janes *et al.*, 1994), and Grb7 (Stein *et al.*, 1994a) have been shown to be involved in Neu signaling pathways (for a review see Dougall *et al.*, 1994). Recently we demonstrated that c-Src tyrosine kinase activity is elevated in Neu-induced mouse mammary tumors and this activation is likely due to the ability of c-Src SH2 domain to interact directly with tyrosine phosphorylated Neu (Muthuswamy *et al.*, 1994, Muthuswamy and Muller, 1995).

The notion that the activation of c-Src tyrosine kinases plays an important role in mammary tumorigenesis is supported by a number of observations. Several studies have shown that human breast cancer specimens possess more than a fourfold increase in c-Src tyrosine kinase activity when compared to normal breast tissues (Rosen *et al.*, 1986; Ottenhoff-kalff *et al.*, 1992) and Neu-induced mouse mammary tumors possess 6–7 fold increase in c-Src tyrosine kinase activity (Muthuswamy *et al.*, 1994). Further support for this hypothesis stems from observations with transgenic expressing PyV middle T antigen in the mammary epithelium (Guy *et al.*, 1992a). Because PyV middle T antigen can associate and activate members of the c-Src family (Courtneidge and Smith, 1983; Kornbluth *et al.*, 1987), mammary epithelial expression of PyV middle T antigen results in a 4–5 fold increase in the tyrosine kinase activities of both c-Src and cYes in mammary tumors (Guy *et al.*, 1994). Indeed, expression of PyV middle T antigen in c-Src deficient mice results in dramatic inhibition of mammary tumor formation (Guy *et al.*, 1994). Further evidence for a role of c-Src in mammary tumorigenesis derives from the recent observation that expression of constitutively active form of c-Src (Y527F) under the control of MMTV promoter/enhancer in transgenic mice results in the formation of mammary epithelial hyperplasias that eventually form mammary tumors (Webster *et al.*, 1995). Taken together, these observations strongly argue that the c-Src tyrosine kinase plays an important role in mammary tumorigenesis.

To analyse whether the other ubiquitously expressed Src family members (c-Yes and Fyn) are involved in Neu-mediated mammary tumorigenesis we measured the *in vitro* kinase activity of c-Yes and Fyn in Neu-induced mouse mammary tumors and adjacent morphologically normal mammary epithelium. Here, we report that c-Yes kinase activity was elevated in Neu-induced mammary tumors when compared to those in the adjacent non-tumorous epithelium. By contrast, Fyn kinase activity was not elevated in Neu-induced mammary tumors. The increase in c-Yes tyrosine kinase activity correlates with its ability to directly associate with Neu in a tyrosine phosphorylation dependent manner *in vivo*. Significantly, although both c-Src and c-Yes associate with Neu, analyses of these immunoprecipitates with antiphosphotyrosine antibodies revealed that c-Src but not c-Yes specifically associates with an 89 kDa phosphotyrosine containing protein (p89). Furthermore, the c-Src associated p89 is found in mammary epithelial tumor cells expressing activated tyrosine kinases but was absent in comparable transformed fibroblast cell lines. Taken together, these data suggest that the p89 associates with c-Src in mammary epithelial cells and not in fibroblasts.

Results

Differential activation of Src family members in Neu-induced mouse mammary tumors

To test whether c-Yes activity was elevated in the Neu expressing mammary tumors, tissue lysates were prepared from mammary tumors and adjacent normal epithelium derived from the MMTV/*neu* transgenic strain. c-Yes was immunoprecipitated using anti-Yes antisera (PAb SK5, Klages *et al.*, 1993) and subjected to *in vitro* kinase assays. To quantitate the level of c-Yes activity the immunoprecipitates were incubated with acid denatured enolase as an external substrate and the radioactivity transferred onto the enolase was measured by PhosphorImager analysis. As shown in Figure 1A, all the lysates derived from mammary tumors (lanes 4, 6, 8, 10 and 12) possessed elevated (3–7-fold) c-Yes tyrosine kinase activity when compared to those derived from adjacent epithelium

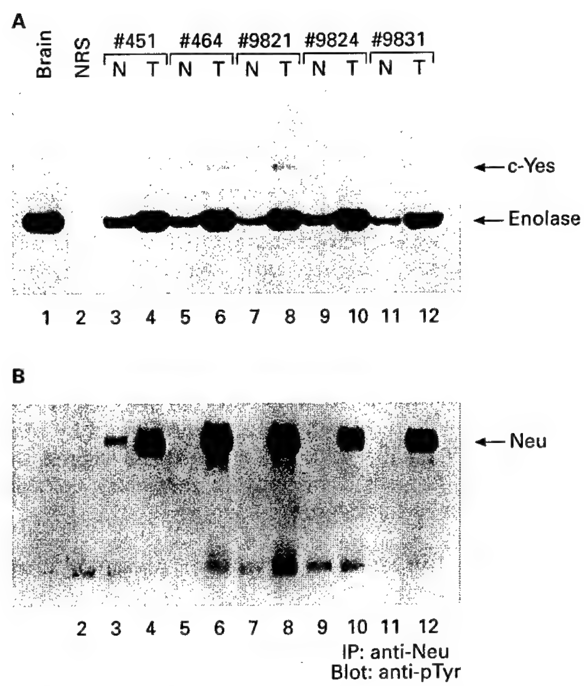

Figure 1 Neu-induced mouse mammary tumors possess elevated c-Yes kinase activity. **(A)** c-Yes was immunoprecipitated using 750 μg of tissue lysate from adjacent normal epithelium (N) or tumor (T) and the immunoprecipitates were incubated with [γ-^{32}P]ATP and acid denatured Enolase. c-Yes immunoprecipitate (lane 1) and a normal rabbit serum (NRS) immunoprecipitate from brain lysate (lane 2) were used as positive and negative controls respectively. The position of Enolase and c-Yes are marked by arrows. **(B)** Neu was immunoprecipitated from the same lysates used in panel A and the immunoprecipitates were immunoblotted with antiphosphotyrosine antibodies. Numbers #451, #464, #9821, #9824, #9831 correspond to mouse ear tag numbers

(lanes 3, 5, 7, 9 and 11). Control immunoblot analysis using Yes-specific antisera suggested that the tumor samples had a 0–3-fold increase in c-Yes protein levels compared to the adjacent normal breast tissues (data not shown). Analysis of an additional eight matched sets of tumor and nontumorous epithelium also showed a consistent increase (2.5–7.5-fold) in c-Yes tyrosine kinase activity (data not shown). The increase in kinase activity showed a tight correlation with the presence of tyrosine phosphorylated Neu (Figure 1B). These observations indicate that c-Yes may play a role in Neu-induced mammary tumorigenesis.

Another Src family member that is known to be widely expressed in various tissue types is the Fyn tyrosine kinase. To test whether Fyn tyrosine kinase activity was elevated in Neu-induced mammary tumors, Fyn was immunoprecipitated from both tumor and adjacent epithelial tissues and incubated with acid denatured enolase and [γ^{32}P]-ATP. Interestingly, the tumor tissues did not display elevated levels of Fyn tyrosine kinase activity compared to that observed in the adjacent normal epithelium (Figure 2A). This was not due to difference in the levels of Fyn protein or due to lack of tyrosine phosphorylated Neu since, control immunoblot analysis using part of the Fyn immunoprecipitates revealed that both tumors and adjacent epithelium possess significant levels of Fyn protein (Figure 2B). Moreover immunoblot analysis of Neu immunoprecipitates with phosphotyrosine antibodies revealed that all the tumor samples possess high levels of tyrosine phosphorylated Neu (Figure 2C). To confirm that it is not a sampling difference, the same tumor lysates were assayed for c-Yes kinase activity. c-Yes immunoprecipitates from the tumors possessed elevated kinase activity when compared to the adjacent nontumorous epithelium (#4144 3.0-fold; #4146 T1 3.0-fold, T2 3.4-fold) (data not shown). By contrast to the observations made with c-Src and c-Yes, Fyn tyrosine kinase activity was higher in the adjacent nontumorous epithelium than that observed in tumor samples (Figure 2A). This difference correlated with the difference in the amount of Fyn protein expressed in the nontumorous epithelium (Figure 2B). At present the significance of this differential expression pattern is unclear. However, these observations suggest that Fyn kinase activity is not elevated in Neu-induced mammary tumors.

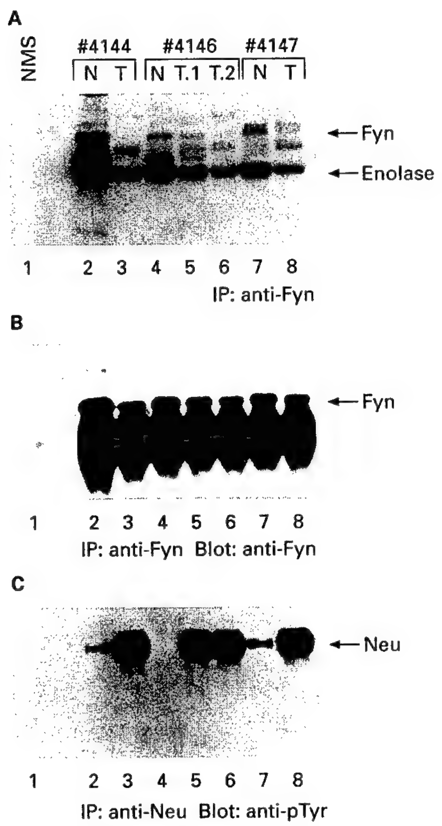

Figure 2 Fyn tyrosine kinase activity is not elevated in Neu-induced mammary tumors. Tissue samples were prepared from both mammary tumors (T) and adjacent nontumorous epithelium (N). **(A)** Fyn was immunoprecipitated from the tissue lysates (750 μg) and a part of the immunoprecipitate was incubated with acid denatured enolase and [γ-^{32}P]ATP. The position of Enolase and Fyn are indicated by arrows. **(B)** The remaining portion of the Fyn immunoprecipitate was resolved on SDS–PAGE and immunoblotted with anti-Fyn antibodies. **(C)** Neu was immunoprecipitated from the same batch of lysates and immunoblotted with anti-phosphotyrosine antibodies (anti-pTyr). A normal mouse serum (NMS) immunoprecipitate from #4144 tumor lysate was used as negative control (lane 1). The numbers #4144, #4146 and, #4147 correspond to mouse ear tag numbers

c-Yes associates with Neu in a tyrosine phosphorylation dependent manner

To understand the mechanism by which c-Yes kinase activity is elevated in Neu-induced mammary tumors, we tested whether c-Yes can associate with Neu in tumor lysates. To this end, c-Yes was immunoprecipitated from tumor lysates and the immunoprecipitates were immunoblotted with anti-Neu antibodies. As shown in Figure 3A, Neu could be detected in the c-Yes immunoprecipitates (lanes 2–4). Because the adjacent nontumorous tissues did not possess any tyrosine phosphorylated Neu (Figure 1) and c-Yes was not found associated with Neu in lysates derived from the adjacent epithelium (data not shown) it is likely that the observed interaction between c-Yes and Neu in mammary tumor lysates requires tyrosine phosphorylated Neu. Consistant with this hypothesis anti-phosphotyrosine immunoblot analysis of c-Yes immunoprecipitates from Neu-induced mouse mammary tumor lysates demonstrated that c-Yes associates with tyrosine phosphorylated Neu (data not shown). To obtain a formal proof for this hypothesis, Rat.2 derived cells lines (NT.12) expressing the activated form of Neu (Val 664 to Glu, Bargmann *et al.*, 1986) under the control of MMTV promoter (Muthuswamy *et al.*, 1994) were utilized. Induction of NT.12 cells with dexamethasone leads to induction of transcription from the MMTV promoter. Since activated Neu possesses 20-fold higher kinase activity than normal Neu (Bargmann and Weinberg, 1988) addition of dexamethasone results in the expression of activated Neu (Figure 3B). The c-Yes protein was immunoprecipitated from both dexamethasone induced and uninduced lysates and blotted with anti-Neu antibodies (Figure 3C). The results indicated that c-Yes was able to coimmunoprecipitate Neu only under conditions where there was tyrosine phosphorylated Neu (Figure 3C, lane 2). The lack of association

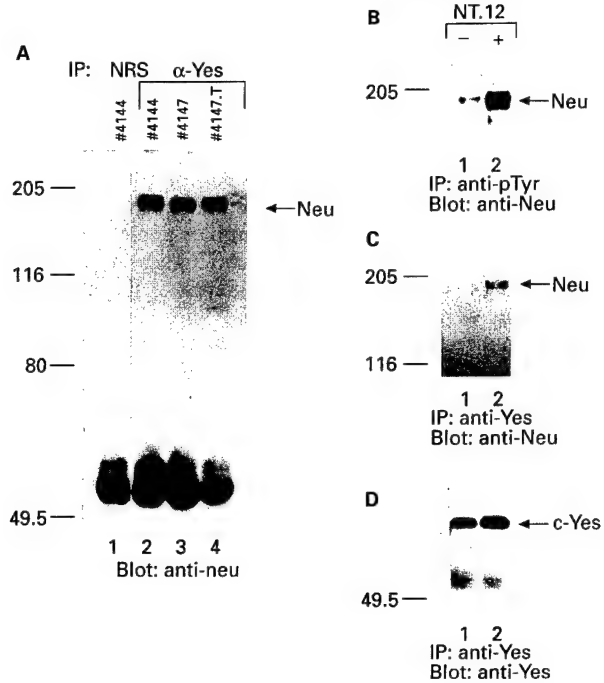

Figure 3 c-Yes associates with Neu in tyrosine phosphorylation dependent manner. (**A**) Tissue lysates were prepared from tumor sample using CHAPS lysis buffer. #4147 tumor was lysed both in CHAPS (lane 3) and TNE (#4147.T, lane 4). c-Yes was immunoprecipitated from 1.5 mg of total lysate and the immunoprecipitate was immunoblotted with anti-Neu antibodies. Normal rabbit serum (NRS) was used as a nonspecific control (lane 1). (**B**) Lysates were prepared from NT.12 cells grown in the absence (−) (lane 1) or presence (+) (lane 2) of dexamethasone. Phosphotyrosine containing proteins were immunoprecipitated from 500 μg of total lysate and immunoblotted with Neu specific antisera. (**C**) c-Yes was immunoprecipitated from 1.5 mg of the same batch of lysates and the immunoblotted with Neu specific antisera. (**D**) The same membrane from (**C**) was reprobed with and antisera that recognizes c-Yes. The molecular weight markers are indicated in kDa

between c-Yes and Neu in the uninduced lysates (Figure 3C, lane 1) was not due to differences in the amount of immunoprecipitable c-Yes protein because control immunoblot of the membrane in Figure 3C with anti-Yes antibodies revealed that there was comparable levels of c-Yes protein under both conditions (Figure 3D, compare lanes 1 and 2). These observations strongly suggest that c-Yes associates with Neu in a tyrosine phosphorylation dependent manner.

c-Yes SH2 domain interacts directly with tyrosine phosphorylated Neu

Since it was clear that c-Yes associates with Neu *in vivo* and this association requires tyrosine phosphorylated Neu it is likely that this interaction is mediated by the c-Yes SH2 domain. *In vitro* analyses using GST fusion proteins containing c-Yes SH2 domain and lysates from tumor tissues and dexamethasone induced and uninduced NT.12 cells demonstrated that the c-Yes SH2 domain alone can interact with tyrosine phosphorylated Neu (data not shown).

Although these observations suggest that c-Yes associates with Neu both *in vivo* and *in vitro*, it is not clear if this association is a direct interaction of the SH2 domain with Neu or mediated by other cellular proteins. To test this possibility, we probed PVDF membranes containing Neu immunoprecipitates with radiolabeled GSTag-c-Yes SH2 fusion protein. As expected, radiolabeled GSTag alone did not bind to any proteins in the total lysate or to the Neu immunoprecipitate (Figure 4A, lanes 1 and 3). However, GSTag-c-Yes SH2 fusion protein bound to Neu both in the total lysate and in the Neu immunoprecipitate (Figure 4B, lanes 1 and 3). The cell lysates used in Figure 4A and B were obtained from a Neu-induced mammary tumor derived cell line NAFA (Muller *et al.*, 1988; Muthuswamy *et al.*, 1994). In order to confirm that this association also requires tyrosine phosphorylated Neu, Neu immunoprecipitates from both dexamethasone induced or uninduced NT.12 cell lysates were probed with radiolabeled GSTag-c-Yes fusion protein. As demonstrated previously treatment of NT.12 cells with dexamethasone induces the expression of tyrosine phosphorylated Neu (Figure 4C, lane 4). An identical membrane was probed with

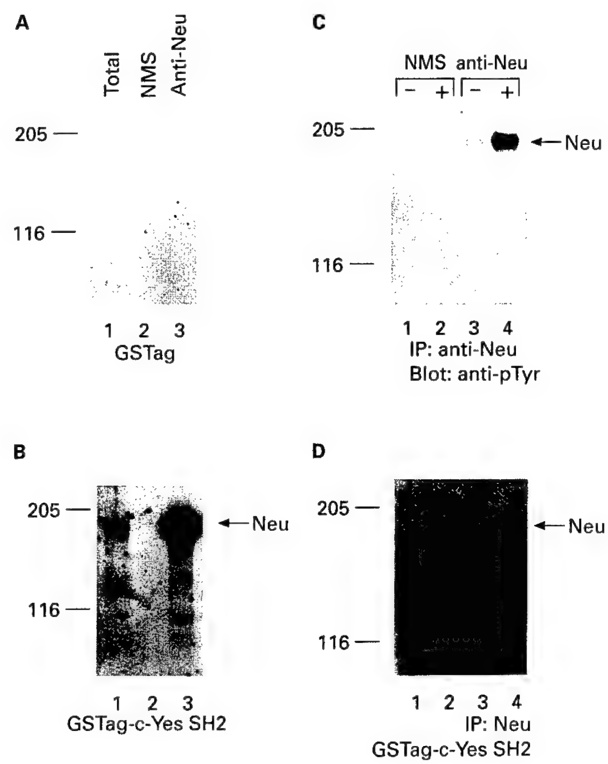

Figure 4 The c-Yes SH2 domain interacts directly with tyrosine phosphorylated Neu. (**A, B**) Total NAFA cell lysate (30 μg, lane 1), normal mouse serum (NMS, lane 2) and an anti-Neu immunoprecipitate (lane 3) from 500 μg of lysate were resolved by SDS–PAGE and transferred onto a membrane. Duplicate membrane strips were probed with either radiolabeled GSTag alone (**A**) or with radiolabeled GSTag-c-Yes SH2 domain (**B**). NT.12 cells were grown both in the presence (+) and absence (−) of dexamethasone. Cell lysates were prepared and Neu was immunoprecipitated. One part of the immunoprecipitate was immunoblotted with anti-pTyr antibodies (**C**) and the other part was probed with radiolabeled GSTag-c-Yes SH2 fusion protein (**D**). Normal mouse serum (NMS) was used as nonspecific control (lanes 1 and 2). The molecular weight markers are indicated in kDa

radiolabeled GSTag-c-Yes SH2 fusion protein (Figure 4D). The results revealed that only the sample harboring tyrosine phosphorylated Neu could directly bind the radiolabeled c-Yes fusion protein.(Figure 4D, lane 4). These observations indicate that c-Yes SH2 domain binds directly to Neu in a tyrosine phosphorylation dependent manner.

The SH2 domains of c-Yes and c-Src associate with the same site on tyrosine phosphorylated Neu and this association is mediated by a phosphorylated tyrosine

Previous observations have shown that c-Src SH2 domain alone can also bind directly to tyrosine phosphorylated Neu (Muthuswamy and Muller, 1995). To determine if c-Yes and c-Src associate with the same site on the Neu molecule we performed an *in vitro* competition assay. GST SH2 fusion proteins containing either c-Yes or c-Src SH2 domains were made in bacteria and purified on a glutathione sepharose column. The c-Src SH2 fusion protein was eluted from the sepharose beads by incubating with free glutathione. The eluted protein is referred to as Sol. GST-c-Src SH2 while the sepharose bead bound fusion protein is referred to as Immobilized (Im.) GST SH2. Cell lysates were derived from either dexamethasone induced or uninduced NT.12 cells. As observed previously, incubation of induced cell lysate containing tyrosine phosphorylated Neu with Im.GST-c-Src SH2 fusion resulted in an association between c-Src SH2 domain and Neu (Figure 5, lane 1) and incubation of uninduced cell lysate did not result in an interaction between c-Src SH2 domain and Neu (lane 2) (Muthuswamy *et al.*, 1994). Competition assay was conducted by preincubating cell lysates derived from dexamethasone induced NT.12 cells with Sol.GST-c-Src SH2 domain fusion protein. These preincubated extracts were subsequently incubated with Im.GST SH2 fusions (Figure 5) of c-Src (lane 3), c-Yes (lane 4) or GAP (lane 5) and the proteins bound to the immobilized SH2 fusions were purified and resolved on SDS–PAGE and immunoblotted with anti-Neu antibodies. As expected, preincubation of cell lysates with Sol. GST-c-Src SH2 fusion protein competes for the ability of Im. GST-c-Src SH2 to associate with Neu (Figure 5, lane 3). Interestingly, preincubation of Sol. GST-c-Src SH2 fusion protein also competed with the ability of Im. GST-c-Yes SH2 domain to associate with Neu (Figure 5 lane 4) implying that both c-Src and c-Yes compete for the same binding site *in vitro*. By contrast, preincubation with Sol. GST-c-Src SH2 had no effect on the ability of Im. GST-GAP SH2 fusion protein to bind to Neu (Figure 5 lane 5, Muthuswamy *et al.*, 1994). These observations suggest that both Src family members (c-Src and c-Yes) are binding the same site on Neu.

In order to confirm that the association between c-Src and c-Yes and tyrosine phosphorylated Neu is a result of a SH2-pTyr interaction we tested whether the SH2 domain-Neu interaction could be competed using commercially available phosphorylated tyrosine as a competing agent. GST SH2 fusions of c-Src and c-Yes were incubated with cell lysates containing tyrosine phosphorylated Neu in the presence of increasing amounts of phosphotyrosine. Proteins bound to the SH2 domains incubated under varying concentrations

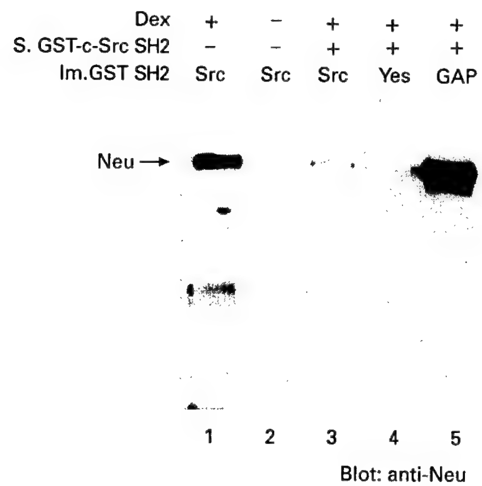

Figure 5 The SH2 domains of c-Yes and c-Src associate with the same site on tyrosine phosphorylated Neu. NT.12 cells were grown in the presence (+) (lanes 1, 3, 4 and 5) or absence (−) (lane 2) of dexamethasone (Dex). 200 μg of lysate was used for lanes 1 and 2 and equal amount (500 μg) of lysates from dexamethasone induced (+) NT.12 cells was used in lanes 3–5. Induced cell lysates (lanes 3–5) were preincubated with the soluble form of GST-c-Src SH2 (Sol.GST-c-Src SH2) and subsequently incubated with immobilized (Im. GST SH2) form of Src (lane 3), Yes (lane 4) or GAP (lane 5) SH2 fusion proteins. Lanes 1 and 2 were incubated with Im. GST SH2 (Src) alone. The proteins bound to the immobilized fusion proteins were resolved by SDS–PAGE and immunoblotted with anti-Neu antibodies

of phosphotyrosine were resolved and probed with anti-Neu antibodies. As shown in Figure 6 the presence of 16 mM phosphotyrosine for c-Src SH2 domain (lane 5) and 50 mM phosphotyrosine for cYes SH2 domain (lane 14) was able to compete with the ability of SH2 domains to interact with Neu while a non-specific competitor such as phosphoserine (50 mM) did not have any effect (Figure 6, lane 1 and 8). This observation was reproducible over three independent experiments. The amount of Neu bound to the SH2 domains under different conditions were quantitated on a phosphorImager. Under conditions where there was no competitor (Figure 6, lane 2 and lane 9) c-Src SH2 domain bound four to five times more Neu than the c-Yes SH2 domain (Figure 6). Six to 8.0 mM of phosphotyrosine was sufficient to impart a 50% decrease in the amount of Neu associtated with c-Src SH2 domain. However, c-Yes SH2 domain required about 38 to 42 mM of phosphotyrosine. These observations suggest two properties of the c-Src and c-Yes SH2 domains, one that the interaction between the SH2 domains and Neu is mediated by a phosphorylated tyrosine on the receptor and second that under these *in vitro* conditions c-Src SH2 domain binds four to five times more Neu molecules albeit with less affinity. The *in vivo* significance of this difference in affinity remains to be tested.

c-Src and c-Yes tyrosine kinases associate with distinct set of tyrosine phosphorylated proteins in vivo

The results of the above analyses and those previously reported (Muthuswamy *et al.*, 1994) suggest that both c-Src and c-Yes tyrosine kinases are involved in Neu-

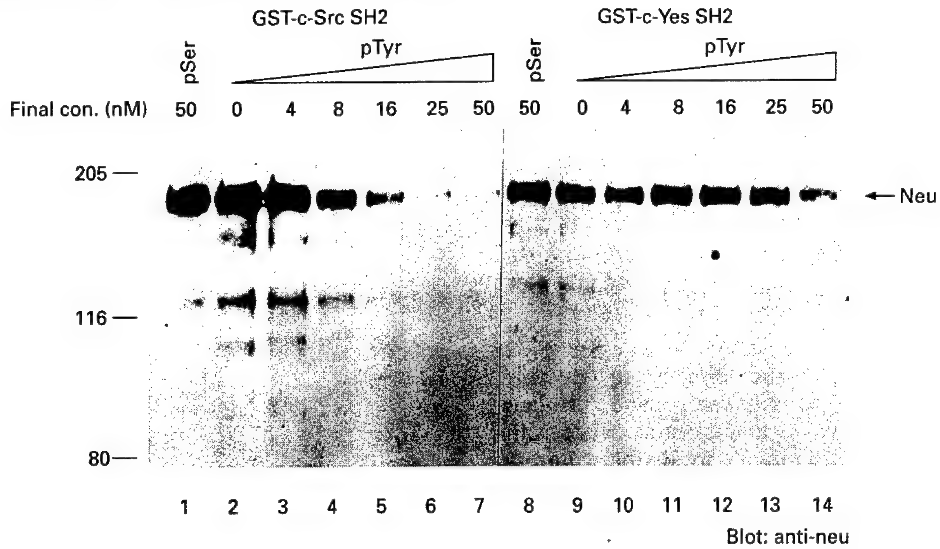

Figure 6 The c-Src and c-Yes SH2-Neu interaction is SH2-pTyr mediated. Purified GST fusion proteins containing c-Src SH2 domain (lanes 1–7) or cYes SH2 (lanes 8–14) were incubated with 500 μg of lysates containing tyrosine phosphorylated Neu either in the presence of no competitor (lanes 2 and 9) or in the presence of increasing amounts of phosphotyrosine (Boehringer Manheim) (lanes 3–7 and 10–14). The fusion proteins were also incubated in the presence of phosphoserine (Boehringer Manheim) (50 mM) as a non-specific control. The proteins bound to the SH2 domains were resolved on a SDS–PAGE and immunoblotted with anti-Neu antibodies. The molecular weight markers are in kDa

mediated mammary tumorigenesis. Although tyrosine phosphorylated Neu could be detected in physical complexes with both c-Src and c-Yes, it was unclear whether c-Src and c-Yes associates with similar or distinct cellular proteins. To explore this possibility, protein extracts from mammary epithelial cell line, NAFA were immunoprecipitated with either c-Src (MAb 7D10) (Figure 7, lanes 3–5) or c-Yes specific antibodies raised against their aminoterminal unique region (Figure 7 lanes 6–8) and subjected to immunoblot analysis with anti-phosphotyrosine antibodies. As expected immunoprecipitation with non-specific control antibodies did not immunoprecipitate any phosphotyrosine containing proteins (lanes 1 and 2). By contrast, immunoprecipitation with either c-Src or c-Yes specific antibodies, revealed a wide spectrum of phosphotyrosine containing proteins associated with these Src family members. The 185 kDa phosphoprotein found in both c-Src and c-Yes immunoprecipitates is likely Neu since it comigrates with Neu in an anti-Neu immunoprecipitate (lane 9). Although both c-Src and c-Yes are associated with Neu, both Src family kinases appear to be associated with a distinct as well as overlapping sets of phosphotyrosine containing proteins. For example, a phosphoprotein of approximately 89 kDa (p89) was consistently found associated with c-Src but was absent in the c-Yes immunoprecipitates. When c-Src immunoprecipitation was carried out in the presence of a competing peptide (aa 2–17) neither c-Src nor p89 was observed in the immunoprecipitate (Figure 8, lane 1). Moreover, a low but detectable amounts of p89 was found associated with c-Src in an anti-Src immunoprecipitate with an independent MAb directed against the c-Src SH3 domain (Ab 327) (data not shown). In addition to p89, several tyrosine phosphorylated proteins between 100–120 kDa were also found associated with c-Src and c-Yes. While the identity of these proteins are unknown, we determined that p89 was not the p80/85 Src substrate (Wu *et al.*, 1991), p85 subunit of PI-3′ kinase or eps8 (97kDa) an EGFR associated phosphoprotein (Fazioli *et al.*, 1993) (data not shown). These

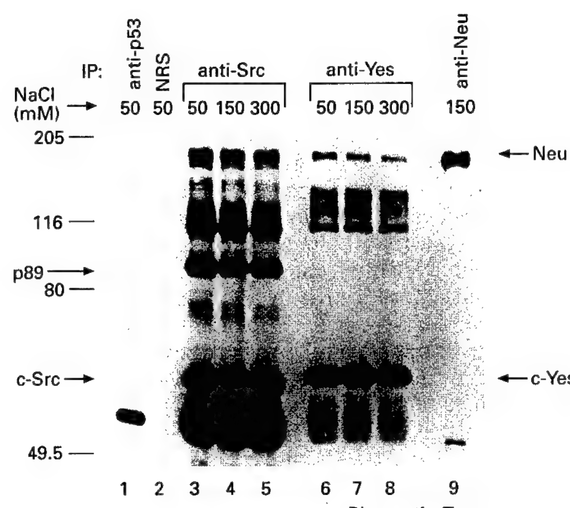

Figure 7 c-Src and c-Yes associate with distinct set of tyrosine phosphorylated proteins *in vitro*. Equal amounts (2.0 mg) of NAFA cell lysates were incubated with anti-Src (lanes 3, 4 and 5) or with anti-Yes (lanes 6, 7 and 8) antibodies. The immunoprecipitates were washed in lysis buffer containing varying concentrations (mM) of NaCl as indicated. Anti-p53 (lane 1) and normal rabbit serum (NRS, lane 2) immunoprecipitates were used as negative controls. An anti-Neu immunoprecipitate (lane 9) using 500 μg of lysate was also used to indicate position of Neu. The immunoprecipitates were immunoblotted with antiphosphotyrosine (anti-pTyr) antibodies. The positions of c-Src, c-Yes, p89 and Neu are indicated. IP: Immunoprecipitate. The molecular weight markers are indicated in kDa

observations suggests that c-Src and c-Yes associate with distinct and novel sets of tyrosine phosphorylated proteins in a Neu-transformed mammary epithelial cell line.

In order to confirm that p89 was not restricted to NAFA cells, we extended this analyses using two independently derived mammary epithelial cell lines that are known to possess elevated c-Src and c-Yes tyrosine kinase activity. Both the mouse mammary epithelial cell line (IA2) derived from a PyV middle T antigen-induced mammary tumor (Guy *et al.*, 1992a; Addison and Graham, unpublished observations) and a human breast cancer cell line (T47D), that is known to express high levels of erbB-2, are also known to possess elevated c-Src tyrosine kinase activity (Muthuswamy *et al.*, 1994). As shown in Figure 8, c-Src immunoprecipitates from NAFA (lane 2), IA2 (lane 3) or T47D cells (lane 4) coimmunoprecipitate a protein that closely migrates with p89 in NAFA cell lysates. However, c-Yes immunoprecipitates from NAFA, IA2 or T47D (lanes 8,9 and 10) did not coimmunoprecipitate p89. The anti-Src antisera were raised against aa 2–17 of the c-Src molecule while the anti-Yes antisera were raised against a GST fusion containing the entire unique region of murine c-Yes (aa 12–78, Klages *et al.*, 1993). To rule out the possibility that our inability to detect association between c-Yes and p89 is due to the anti-Yes antisera used in these assays, we raised Yes-specific antisera (yab-2) against the mouse c-Yes protein using a peptide containing aa 4–20 in the amino terminal unique region of the molecule. Immunoprecipitation of c-Yes from NAFA cells using yab-2 followed by an anti-phosphotyrosine immunoblot clearly showed that p89 was not associated with c-Yes immunoprecipitates (lane 13).

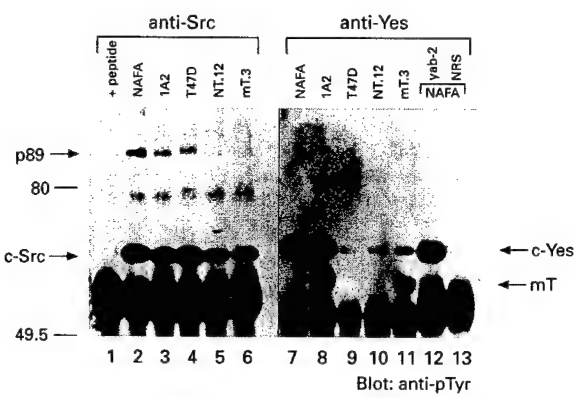

Figure 8 c-Src associates with p89 in transformed epithelial cells but not in transformed fibroblasts. Cell lysates were prepared from transformed mammary epithelial cells NAFA (lanes 1, 2, 7, 12 and 13), 1A2 (lanes 3 and 8) and T47D (lanes 4 and 9) or from transformed fibroblasts, NT.12 (lanes 5 and 10) and mT.3 (lanes 6 and 11) (see text for detailed descriptions of these cell lines). An equal amount (750 μg) of each cell lysate was incubated with c-Src specific (lanes 1–6) or c-Yes specific (lanes 7–12) antisera. The immunoprecipitates were immunoblotted with antiphosphotyrosine (anti-pTyr) antibodies. The anti-Src antibody was preincubated on ice for 20 min with 5–10 μM of aa 2–17 Src peptide prior to the addition of cell lysate (+peptide, lane 1). yab-2 is a Yes specific antisera directed against aa 4–20 of mouse c-Yes protein (lane 12). NRS: normal rabbit serum. The positions of c-Src, c-Yes, PyV middle T antigen (mT) and p89 are indicated. Molecular weight are in kDa

This analysis also proved that yab-2 is specific for c-Yes since, the immunoprecipitates did not bring down any protein that comigrates with c-Src (compare lane 2–6 with 13). To determine if c-Src and c-Yes associated with similar sets of proteins in all cell types transformed by Neu or PyV middle T, we immunoprecipitated c-Src and c-Yes from Neu (NT.12, lanes 5 and 10) or PyV middle T (mT.3, lanes 6 and 11) antigen transformed Rat fibroblasts. Interestingly, under conditions where c-Src (lane 6) and c-Yes (lane 11) can associate with middle T antigen and Neu we were unable to detect any association with p89 in either NT.12 or mT.3 cell lines. Taken together, these observations argue that p89 associates with c-Src in mammary epithelial cells and not fibroblasts.

Discussion

We have presented evidence that strongly suggests that among the ubiqutiously expressed Src family members (c-Src, c-Yes and Fyn) only c-Src and c-Yes kinase activity is elevated in Neu-induced murine mammary tumors. The increase in c-Yes kinase activity correlates with its ability to associate with tyrosine phosphorylated Neu *in vivo*. This *in vivo* association is likely due to the ability of c-Yes SH2 domain to interact directly with Neu in a tyrosine phosphorylation dependent manner as shown *in vitro*. Interestingly, although the tyrosine kinase activity of both c-Src (Muthuswamy *et al.*, 1994) and c-Yes are elevated in Neu-induced mammary tumors, they associate with distinct sets of tyrosine phosphorylated cellular proteins.

The observation that c-Yes kinase activity is elevated in Neu-induced mammary tumors parallels the observation that Neu-induced mammary tumors possess elevated c-Src kinase activity (Muthuswamy *et al.*, 1994). Interestingly, unlike these two Src family members Fyn tyrosine kinase activity was not elevated in Neu-induced mammary tumors. Consistent with this observation, PyV middle T antigen-induced mammary tumors fail to demonstrate increased Fyn tyrosine kinase activity (Guy *et al.*, 1994). Taken together these results argue that activation of Fyn may not be involved in transformation of the mammary epithelium. Interestingly, despite the lack of elevated Fyn kinase activity, complexes between Fyn and either PyV middle T (Cheng *et al.*, 1988; Kypta *et al.*, 1988) or Neu (data not shown) can be detected. Indeed c-Src and c-Yes, but not Fyn activity was elevated in colon carcinoma derived cell lines and primary colon cancers when compared to untransformed cells (Park *et al.*, 1993). Taken together these observations suggests that unlike cSrc and c-Yes, activation of Fyn tyrosine activity may not be involved in these epithelial tumors.

The increase in c-Yes tyrosine kinase activity in Neu-induced mammary tumors is likely due to the ability of c-Yes to directly associate with Neu in tyrosine phosphorylation dependent manner (Figures 3 and 4). These observations are consistent with those observed for PDGFR and CSF-1R which are known to activate c-Yes (Twamley *et al.*, 1992; Courtneidge *et al.*, 1993). Recently we have shown that the increased c-Src kinase activity observed in Neu-induced tumors also results from the ability of c-Src SH2 domain to directly interact with Neu in a tyrosine phosphoryla-

tion dependent manner (Muthuswamy and Muller, 1995). *In vitro* competition analysis suggest that both c-Src SH2 domain and c-Yes SH2 domain bind to the same site on Neu (Figure 5) indicating that the binding of cSrc and c-Yes to Neu are likely mutually exclusive. This *in vitro* association can be competed by phosphotyrosine (Figure 6) suggesting that both c-Src and c-Yes SH2 domains associate with a phosphorylated tyrosine on the receptor. Indeed binding and activation of Src family members by PDGFR requires tyrosines 579 and 581 of the PDGFR (Mori *et al.*, 1993). Although the site of interaction between the Src family members and Neu is not known, it is likely that Neu activates both c-Src and c-Yes by a similar mechanism.

Recent studies have shown that microinjection of antibodies that recognize Src, Yes and Fyn kinases abolishes EGF and PDGF-induced S phase entry of fibroblasts (Twamley-Stein *et al.*, 1993; Roche *et al.*, 1995). These observations indicate that Src family members are required for PDGF and EGF induced entry into cell cycle. However the importance of elevated c-Src and c-Yes kinase activity in Neu-induced mammary tumors remains to be ascertained. Future interbreeding between MMTV/*neu* transgenics and c-Src or c-Yes deficient mice would allow this question to be addressed.

Our results further suggest that in transformed mammary epithelial cells c-Src and c-Yes associates with distinct and overlapping sets of tyrosine phosphorylated cellular proteins. It is likely that the higher molecular weight Src and Yes associated proteins may include Src substrates such as ppl25 (FAK) (Cobb *et al.*, 1994; Xing *et al.*, 1994), pll0, pl20 or pl30 (Kanner *et al.*, 1990, 1991; Reynolds *et al.*, 1992). Interestingly, a prominent protein with an approximate molecular weight of 89 kDa was observed only in c-Src immunoprecipitates from transformed epithelial cell lysates (Figures 7 and 8). However, c-Src did not show any association with p89 in Neu or PyV middle T antigen transformed fibroblast cells. These observations suggest that p89 interacts with c-Src only in mammary epithelial cells and not in fibroblasts.

The notion that c-Src and c-Yes may function differently is supported by a number of observations. For example, expression of MMTV/PyV middle T antigen in the absence of c-Src results in dramatic reduction in induction of mammary tumors in transgenic mice whereas expression of MMTV/middle T in the absence of c-Yes has no effect on the ability of middle T to induce mammary tumorigenesis (Guy *et al.*, 1994). These data argue that PyV middle T antigen specifically requires the presence of c-Src for transformation of the mammary epithelium whereas c-Yes is dispensable for this process. It is conceivable that the c-Src-specific association of mammary epithelial proteins such as p89 is involved in this tissue specifity. However, PyV middle T antigen can transform either fibroblast or endothelial cells lacking c-Src (Kiefer *et al.*, 1994; Thomas *et al.*, 1993). Interestingly, PyV middle T antigen-mediated transformation of endothelial cells is severely impaired in c-Yes deficient mice whereas tumor formation remains unaffected in the c-Src deficient mice (Thomas *et al.*, 1993; Kiefer *et al.*, 1994). These observations suggest that transformation mediated by c-Src and c-Yes is dependent on cell type. Furthermore, mice lacking c-Src develop osteopeterosis due to defective osteoclast resorption (Soriano *et al.*, 1991; Lowe *et al.*, 1993) whereas mice lacking functional c-Yes have no overt phenotype (Stein *et al.*, 1994b). Taken together these observations suggest that c-Src and c-Yes have tissue specific function. Given the observation that p89 is specific to c-Src and not c-Yes, the future identification of p89 may provide novel insights into the mechanisms involved in c-Src-mediated transformation of the mammary epithelium.

Materials and methods

DNA constructs and antibodies

The bacterial fusion protein bearing the SH2 domain of c-Yes (amino acids 141 to 266) fused to glutathione-S-transferase (GST) was a generous gift of B Rowley and J Bolen (Bristol-Myers Squibb, New Jersey). The GAP SH2 fusion DNA contains both SH2 domains flanking the SH3 domain (amino acids 181 to 451) and was inserted as an Eco R1 fragment in pGEX 3X (Muthuswamy *et al.*, 1994). The GSTag SH2 fusion for c-Yes was constructed by subcloning a BamHI/EcoRI fragment containing the SH2 domain from GST-cYes SH2 into a BamHI/EcoRI site in pGSTag (Ron and Dressler, 1992). The construction of MMTV promoter/enhancer activated Neu fusion gene (pMMTV/Neu NT) has been described previously (Muller *et al.*, 1988). Anti-Yes (PAb SK5, Klages *et al.*, 1993) is a generous gift of B Rowley and J Bolen. AntiYes antisera, yab-2, was generated by using a 16 amino acid peptide (aa 4–20 murine c-Yes, IKSKEKSPAIKYTPEN) coupled to KLH (synthesized at Mobix central facility, McMaster University). Other antisera used are anti-Fyn (Santa Cruz Biotechnology Inc.), anti-Neu (MAb.3, Oncogene Science), anti-Yes antibodies that cross reacts with both Src and Yes (generous gift of Marius Sudol), anti-phosphotyrosine (MAb 4G10, Upstate Biotechnology, Inc.).

Cell lines

Rat 2 fibroblast cell line expressing activated *neu* under MMTV transcriptional control (NT.12) was established by transfecting pMMTV/*neu* NT plasmid and is described elsewhere (Muthuswamy *et al.*, 1994). To induce the expression of activated *neu*, the NT.12 cells were grown in the presence of dexamethasone (final concentration 10^{-6} M) for a period of 24 to 36 h. Fibroblast cells expressing PyV middle T antigen was derived by transfecting Rat 2 cells with pSV2middleT plasmid. G418 resistant clones were selected and a clone mT.3 was used for this study. Mouse mammary epithelial cell line NAFA was derived from an activated *neu*-induced mouse mammary tumor (Muller *et al.*, 1988; Muthuswamy *et al.*, 1994) and was grown in modified DMEM containing 10% fetal bovine serum. The mammary epithelial cell line 1A2 was derived from a PyV middle T antigen-induced mouse mammary tumor (provided by Christina Addison and Frank Graham), and was grown in RPMI containing 10% fetal bovine serum and 1× GMS-X (Gibco BRL). The human breast cancer derived cell line T47D was obtained from ATCC and was cultured as recommended. All cell lines were grown in 100 mm plates to confluency ($5-6\times10^6$ cells) prior to lysis.

Immunoprecipitation and in vitro *kinase assays*

Tissue samples were ground to a powder under liquid nitrogen and lysed in TNE lysis buffer (50 mM Tris HCl

pH 7.6; 150 mM NaCl; 1% NP40; 2 mM EDTA; 1 mM sodium orthovanadate; 10 $\mu g\ ml^{-1}$ leupeptin; 10 $\mu g\ ml^{-1}$ aprotinin). Cells were lysed in TNE lysis buffer only when the lysates were used for *in vitro* kinase assays, whereas for all coimmunoprecipitation analyses cells were lysed in low salt CHAPS lysis buffer (50 mM Tris (pH 8.0); 0.7% CHAPS; 50 mM NaCl; 1 mM sodium orthovanadate, 10 $\mu g\ ml^{-1}$ leupeptin; 10 $\mu g\ ml^{-1}$ aprotinin; 5 $\mu g\ ml^{-1}$). Lysis was performed for 20 to 25 min on ice and the lysates were cleared by centrifuging at 12 000 *g* at 4°C for 20 min. Fresh lysates were used for all coimmunoprecipitation studies. Coimmunoprecipitations were performed by incubating 1.0 to 2.0 mg of the protein lysate with 0.5 to 1.0 μg of respective antisera, 30 to 40 μl of protein G Sepharose fast flow (Pharmacia) in a final volume of 500–600 μl for 2.0 h at 4°C on a rotating platform. The precipitates were washed four to five times with respective lysis buffer and used for further analysis. In some instances, where mentioned, NaCl concentration of the lysis buffer used for wash was modified.

For *in vitro* kinase assays c-Yes or Fyn immunoprecipitates were washed once with 2× kinase buffer (200 mM HEPES pH 7.0; 10 mM $MnCl_2$) and were resuspended in 20 μl of mix containing 10 μl of 2× kinase buffer; 1 μl of mix containing 10 μl of 2× kinase buffer; 1 μl of [γ-^{32}P]ATP (>45000 Ci $mmol^{-1}$, 1.0 mCi ml^{-1} Dupont, NEN) and 10 μg of acid denatured enolase in 10 μl. The reaction was terminated by adding an equal volume of 2× SDS gel loading buffer (62.5 mM Tris HCl pH 6.8; 2% SDS; 5% glycerol; 0.7 M 2-mercaptoethanol; 0.25% bromophenol blue). The samples were electrophoresed on a 10% SDS-polyacrylamide gel, the gels were incubated in 1 M KOH for 30 to 45 min at 45°C, dried and subjected to autoradiography. The dried gels were quantitated by phosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Immunoblotting

The immunoprecipitates were resuspended in SDS gel loading buffer and the proteins were resolved on a 9% SDS polyacrylamide gel. The proteins were transferred onto a PVDF membrane (Millipore) using an immunoblot transfer apparatus (BioRad). Following an overnight incubation in 3% skim milk in 1× PBS at 4°C, the membrane was incubated with either antiFyn (1:750, Santacruz), or anti-Neu (Ab-3, 1:1000, Oncogene Sci.), anti-Yes (1:1000) in 3% skim milk for 3 h at room temperature. The membranes were washed five times with 1×PBS for 5–10 min each and were incubated with 1:5000 dilution of anti-mouse IgG or anti-rabbit IgG, conjugated with HRP (BioCan Scientific) for one hour. The membranes were washed and the proteins were visualized by enhanced chemiluminescence (ECL) system (Amersham). [^{125}I] IgG was used whereever quantitation was necessary.

Anti-phosphotyrosine immunoblots were performed in a similar fashion with the exception that the membranes were blocked overnight in 3% BSA (Sigma) in TBS (20 mM Tris.HCl pH 7.5; 150 m NaCl; 5 mM KCl) and probed for 3 h with anti-phosphotyrosine antibodies (4G10, 1:750, UBI) in 3% BSA in TBS. After washing the blots in TBS; 0.05% Tween 20 (TBS-T), the blot was incubated in 3% milk in TBS for 1 h. The membrane was incubated with HRP conjugated anti-mouse IgG (1:5000) for 45–60 min and the membranes were washed twice with TBS-T for 10 min followed by three 5 min wash with TBS alone. The proteins were visualized by the ECL detection system (Amersham).

In vitro *analyses using GST fusion proteins*

Preparation of fusion proteins, *in vitro* associations studies, *in vitro* competition analyses and direct binding assays using GSTag-SH2 fusion proteins were performed as described earlier (Muthuswamy *et al.*, 1994; Muthuswamy and Muller, 1995).

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Biochemistry

Mutations affecting conserved cysteine residues within the extracellular domain of Neu promote receptor dimerization and activation

(receptor tyrosine kinase/transformation)

Peter M. Siegel*† and William J. Muller*‡§

*Institute for Molecular Biology and Biotechnology and Departments of †Biology and ‡Pathology, McMaster University, Hamilton, ON, Canada, L8S 4K1

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ABSTRACT **Overexpression of the Neu/ErbB-2 receptor tyrosine kinase has been implicated in the genesis of human breast cancer. Indeed, expression of either activated or wild-type *neu* in the mammary epithelium of transgenic mice results in the induction of mammary tumors. Previously, we have shown that many of the mammary tumors arising in transgenic mice expressing wild-type *neu* occur through somatic activating mutations within the *neu* transgene itself. Here we demonstrate that these mutations promote dimerization of the Neu receptor through the formation of disulfide bonds, resulting in its constitutive activation. To explore the role of conserved cysteine residues within the region deleted in these altered Neu proteins, we examined the transforming potential of a series of Neu receptors in which the individual cysteine residues were mutated. These analyses indicated that mutation of certain cysteine residues resulted in the oncogenic activation of Neu. The increased transforming activity displayed by the altered receptors correlated with constitutive dimerization that occurred in a disulfide bond-dependent manner. We further demonstrate that addition of 2-mercaptoethanol to the culture medium interfered with the specific transforming activity of the mutant Neu receptors. These observations suggest that oncogenic activation of Neu results from constitutive disulfide bond-dependent dimerization.**

Comma

Oncogenic activation of growth factor receptors can be mediated by the overexpression of either the ligand or receptor, as well as mutations that promote ligand-independent dimerization or catalytic activation. The Neu receptor tyrosine kinase is one such growth factor receptor that has been shown to be activated by several of these mechanisms (1). Neu is a member of the epidermal growth factor receptor (EGFR) family, which includes the EGFR (ErbB-1), Neu (ErbB-2), ErbB-3, and ErbB-4 receptor tyrosine kinases (2–7). Although a specific ligand that binds directly to Neu has yet to be cloned, several reports suggest that a biochemical activity known as the Neu activating factor may be one such candidate (8, 9). Activation of Neu can occur through the formation of heterodimers with other members of the EGFR family following stimulation with ligands specific for EGFR, ErbB-3, and ErbB-4 (10–14). Overexpression of the wild-type Neu receptor has also been reported to overcome the need for ligand activation and increases the transforming ability of the receptor (15). Finally, mutational activation of Neu has been described; both point mutation (16) or deletion of the entire extracellular domain constitutively activates the receptor (17). The best characterized of these mutations is the transmembrane point mutation that converts a valine residue to glutamic acid at amino acid position 664 (16). This particular mutation has been shown to increase the kinase activity of Neu (18–20) by promoting receptor dimerization (21).

More recently, we have described the occurrence of novel mutations within the extracellular domain of Neu in mammary tumors derived from transgenic mice expressing a mouse mammary tumor virus (MMTV)/wild-type *neu* fusion gene (22, 23). Although these experiments demonstrated that the mutations occurring within the *neu* transgene were capable of increasing the transforming ability of Neu, the precise molecular mechanism by which this occurs has yet to be addressed. Because the well-characterized point mutation in the transmembrane domain (16) has been shown to induce receptor dimerization (21), we examined the possibility that these mutations within the extracellular domain could function in a similar manner. Here we demonstrate that dimers of these altered Neu receptors can be detected when immunoprecipitates are separated under nonreducing conditions but are undetectable when a reducing agent is present. Because many of the deletions affect specific conserved cysteine residues in the juxtatransmembrane region of Neu, we investigated the importance of these cysteine residues by creating a series of point mutations and measuring their specific transforming activity in Rat-1 cells. Like deletions, certain mutations affecting these cysteine residues were capable of activating the transforming ability of Neu by promoting receptor dimerization. The importance of disulfide bonding in receptor dimerization was further reinforced by the observation that the transforming activity of these mutant Neu molecules was impaired by the addition of reducing agents. Taken together, our observations suggest that oncogenic activation of these Neu mutants involves constitutive dimerization of the receptor through the formation of cysteine disulfide bonds.

MATERIALS AND METHODS FN1

DNA Constructs. Generation of the pJ4Ω plasmids containing various forms of the rat *neu* cDNA have been described (22). The cysteine mutations were created by polymerase chain reaction (PCR) following previously described methods (22, 24). The resulting PCR products containing the various cysteine mutations were cloned into the wild-type *neu* cDNA as *Bst*1107I/*Eag*I fragments. The amplified regions were sequenced to ensure that only the desired mutations were present. A plasmid (PGK-puro) containing the puromycin resistance gene under the transcriptional control of the ~~phos-phoglyceraldehyde~~ phosphoglycerate kinase promoter was a generous gift of Michael A. Rudnicki (●●). The plasmid pJ4ΩmT was kindly provided by Marc A. Webster (●●) and was constructed by releasing the polyomavirus middle T antigen cDNA from the plasmid

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Abbreviations: EGFR, epidermal growth factor receptor; MMTV, mouse mammary tumor virus.
§To whom reprint requests should be addressed.

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B McMaster University, Hamilton, Ontario

C McMaster University, Hamilton, Ontario

1 The shading of the cysteine residues appear very dark. I have included another version of Fig. 1 in which the shading is lighter.

pMMTV MT (25) as a *Hin*dIII-*Eco*RI fragment and inserting it into the corresponding sites of pJ4Ω.

Cell Lines and Focus Assays. Rat-1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and fungizone. Stable cell lines expressing the Neu cysteine mutants were derived by electroporation as described (22). DNA was introduced into the Rat-1 cells at a 10:1 ratio of expression plasmid to puromycin resistance plasmid (PGK-puro) and resistant colonies were selected in media containing 3.0 μg of puromycin per ml. The focus assays were performed as previously outlined (22). When 2-mercaptoethanol was included, the cells were allowed to reach a monolayer prior to adding the reducing agent. The monolayer was maintained for 12 days in DMEM supplemented with 5% fetal bovine serum, penicillin, streptomycin, and fungizone.

Immunoprecipitations and Immunoblotting. Detection of dimer formation between Neu receptors harboring either deletions or cysteine point mutations was performed as follows. Confluent 100-mm plates of Rat-1 cells expressing the various forms of Neu were washed twice with ice cold 1× PBS containing 10 mM iodoacetamide. The cells were lysed for 20 min on ice in TGP buffer (1% Triton X-100/10% glycerol/50 mM Hepes, pH 7.4/1 mM sodium orthovanadate/10 μg of aprotinin per ml/10 μg of leupeptin per ml) containing 10 mM iodoacetamide. Lysates were cleared by centrifugation at 4°C for 10 min, and protein concentrations were determined by the Bradford assay (Bio-Rad). Immunoprecipitations were carried out in duplicate by using 200 μg of total protein that was incubated with anti-Neu antibodies (7.16.4) conjugated to protein G-Sepharose beads (Pharmacia). Immunoprecipitations were carried out at 4°C for 3–4 h with gentle agitation. Neu immunoprecipitates were washed three times in Ripa lysis buffer (1% Triton X-100/0.1% SDS/1% sodium deoxycholate/10 mM $NaPO_4$/150 mM NaCl/2 mM EDTA/50 mM NaF/1 mM sodium orthovanadate/10 μg of aprotinin per ml/10 μg of leupeptin per ml) and one set was resuspended in SDS gel loading buffer (62.5 mM Tris·HCl, pH 6.8/2% SDS/5% glycerol/0.25% bromophenol blue) containing 0.8 M 2-mercaptoethanol while the duplicate set was resuspended in SDS gel loading buffer lacking any reducing agent. Immunoprecipitates that were subjected to either reducing or nonreducing conditions were boiled for 10 min, and the proteins were separated by electrophoresis through 4–12% gradient SDS polyacrylamide gels. The proteins were transferred and the membranes probed as described (26).

RESULTS

Mutations Within the *neu* Protooncogene Are Clustered Within a Cysteine Rich Region of the Extracellular Domain That Is Conserved Among the Four EGFR Family Members. The mutations initially discovered in tumors arising in MMTV/wild-type *neu* transgenic mice reside in the extracellular domain of the Neu receptor itself (22). These altered receptors are capable of mediating transformation as measured by focus forming assays in Rat-1 fibroblasts (ref. 21; Fig. 1*A*). Alignment of these deletions revealed that they affect three of five cysteine residues located in the juxtatransmembrane region of Neu. In the one case where all five cysteine residues were retained (NeuX3, Fig. 1*A*), the deletion removed a conserved proline residue immediately adjacent to the cysteine residue proximal to the transmembrane domain. We have also isolated an example of an insertion mutation involving the in-frame insertion of 3 amino acids (Neu8564). Significantly, one of the inserted amino acids in this transforming Neu mutant was a cysteine residue (Fig. 1*A*). The importance of these cysteine residues is further reinforced by the observation that all five cysteine residues located within this region are conserved among the other three EGFR family

ref. 22
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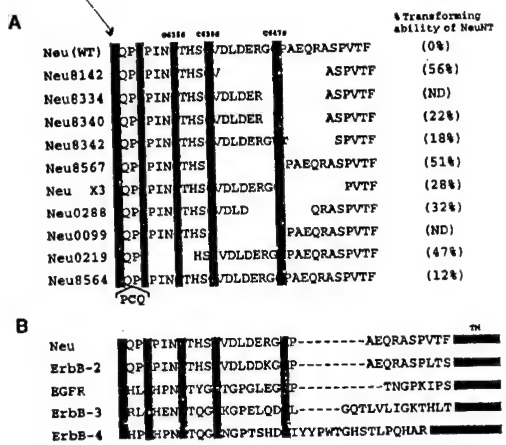

FIG. 1. The region of Neu containing the identified deletions displays limited homology among the other members of the EGFR family. (*A*) Protein sequence alignment of the deletions within Neu that have been cloned and sequenced (22). The transforming abilities of the various altered receptors were assessed by focus forming assays in Rat-1 cells and are expressed as percentage transformation compared with a well-characterized transforming mutant of Neu (NeuNT; 16). The values listed represent the average obtained from three independent focus assays. The transforming activities for Neu8142, Neu8340, Neu8342, and Neu8567 have been reported (22). ND, not determined. (*B*) Protein sequence alignment of the four members of the EGFR family. Both the rat (Neu) and human (ErbB-2) sequence is included for Neu/ErbB-2, while the human sequence alone is presented for EGFR, ErbB-3, and ErbB-4. The dashes have been included for the purposes of this alignment (adapted from ref. 7). The black box represents the position of the transmembrane domain (TM).

members (Fig. 1*B*). Taken together, these observations suggest that alteration of these cysteine residues, either by deletion or mutation, may be involved in the activation of the Neu receptor tyrosine kinase.

The Presence of Deletion Mutations Within *neu* Promotes Receptor Dimerization. Because previous studies have demonstrated that activated Neu receptors bearing the single point mutation in the transmembrane domain were activated due to constitutive dimerization (21), we were interested in assessing whether the altered Neu receptors harboring these deletions were also capable of dimer formation. To accomplish this, stable cell lines expressing selected mutant receptors were generated (22). Neu was immunoprecipitated from these cell lines and separated on SDS/polyacrylamide gels under both non-reducing and reducing conditions. As shown in Fig. 2*A*, Neu immunoprecipitates separated under nonreducing conditions revealed the presence of Neu dimers in those cell lines expressing mutant receptors (lanes 3–6). In contrast, stable dimer formation could not be detected in a cell line overexpressing the wild-type Neu receptor (N17; lane 2) The absence of dimer formation in wild-type Neu-expressing cells was not the result of decreased protein levels since both mutant and wild-type Neu-expressing cells possessed comparable levels of Neu (Fig. 2*B*). Interestingly, the extent of dimer formation observed for the different Neu deletion mutants was directly correlated with their transforming activity (compare Fig. 1 and Fig. 2). For example, a larger percentage of the higher transforming mutant, 8567, was in the form of dimers when compared with the weaker transforming mutant, 8342, despite the fact that both cell lines expressed similar levels of Neu protein (Fig. 2*A*, lanes 5 and 6). To explore whether receptor dimerization required disulfide bonding, duplicate immunoprecipitates were subjected to immunoblot analysis for Neu

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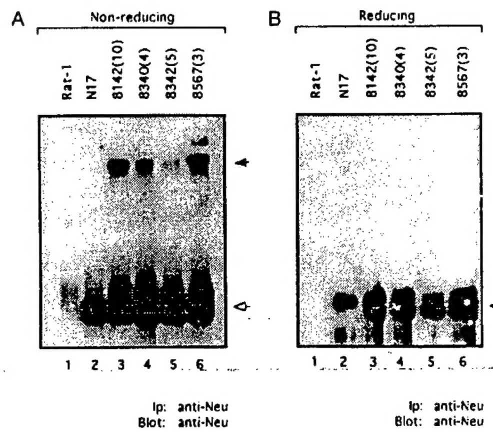

FIG. 2. Neu receptors harboring deletions undergo constitutive dimerization. Immunoprecipitates (IP) from stable Rat-1 cell lines expressing either wild-type Neu (N17) or selected altered forms of Neu (8142, 8340, 8342, 8567) were electrophoresed through 4–12% gradient SDS/polyacrylamide gels under nonreducing (*A*) or reducing (*B*) conditions. Proteins were transferred to polyvinylidene difluoride membranes and subjected to immunoblot analysis (blot) with antibodies specific to Neu (anti-Neu). (*A*) Neu dimers are indicated by the solid arrowhead, while the position of the monomer is marked by the open arrowhead. (*B*) The position of monomeric Neu is indicated by the open arrowhead. In both *A* and *B*, the lane marked Rat-1 indicates the parental cell line that does not contain an expression vector.

after separation under reducing conditions (Fig. 2*B*). The results revealed that under these reducing conditions only the monomeric form of Neu was detected (Fig. 2*B*). These results provide evidence that deletions within this region of Neu promote the formation of disulfide-linked dimers.

Point Mutations Affecting Particular Conserved Cysteine Residues Can Induce Neu-Mediated Transformation. To directly test the importance of the conserved cysteine residues in activating the transforming potential of Neu, we mutated individual cysteine residues located at positions 635, 639, and 647. These residues were selected for mutagenesis because each of these cysteines was removed by at least one of the deletions (Fig. 1*A*). Initially we constructed expression vectors containing mutant *neu* molecules in which these individual cysteine residues were converted to serine residues (C635S, F3 C639S, and C647S; Fig. 3*A*). Analysis of the transforming activity of these altered Neu receptor tyrosine kinases in Rat-1 cells revealed that conversion of cysteine 635 or 647 to a serine residue resulted in activation of the transforming potential of T1 Neu (15% and 12% of NeuNT, respectively; Table 1). However, mutation of cysteine 639 to a serine residue (C639S) resulted in only a weak stimulation of transforming activity (2% of NeuNT; Table 1). To ensure that it was the loss of the cysteine residues that was critical for receptor activation, the cysteine at amino acid position 635 was also converted to either a glycine or a methionine (C635G and C635M; Fig. 3*A*). The substitution of this cysteine with either residue resulted in transformation of Rat-1 cells to ≈14% and 13% of NeuNT respectively (Table 1). To confirm that the altered Neu receptors promote transformation, stable cell lines individually expressing the four deletion mutants and three cysteine mutants described in this study were tested for their ability to grow in soft agar. In contrast to the parental Rat-1 line or cells overexpressing normal Neu (N17), all of the cell lines harboring mutant versions of Neu formed colonies in soft agar with varying efficiencies (data not shown). Finally, transgenic mice

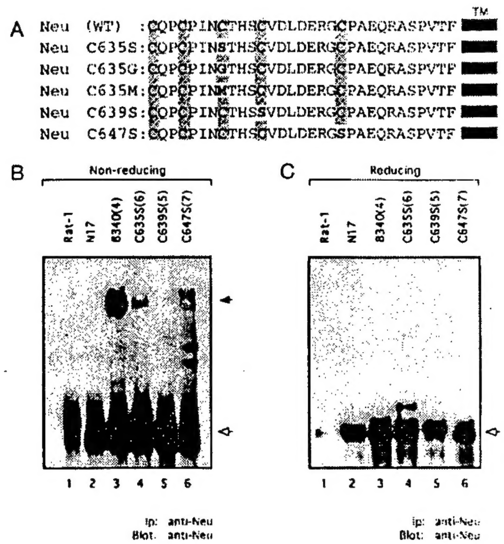

FIG. 3. Individual alteration of conserved cysteine residues in Neu promotes receptor dimerization. (*A*) Protein sequence alignment of the wild-type Neu sequence and each of the five cysteine mutations. The residues outlined in gray highlight the conserved cysteine residues in this region of Neu. The amino acids that have been substituted for the indicated cysteine residues are denoted by boldface type. The black box following the sequence in each case represents the start of the transmembrane domain (TM). (*B* and *C*) Neu was immunoprecipitated (IP) from stable Rat-1 cell lines expressing either wild-type Neu (N17) or cell lines expressing Neu cysteine mutants (C635S, C639S, C647S). The immunoprecipitates were separated on 4–12% gradient SDS/polyacrylamide gels under nonreducing (*B*) or reducing (*C*) conditions. Proteins were transferred to polyvinylidene difluoride membranes and subjected to immunoblot analysis with Neu-specific antibodies (anti-Neu). Dimers of the Neu receptor are indicated by the filled in arrowhead while the position of the monomer is marked by an open arrowhead. The lanes designated Rat-1 indicate immunoprecipitates from the parental cell line that does not contain an expression vector.

have been derived which overexpress either the 8142 or 8342 deletion (Fig. 1*A*) in the mammary gland under the control of the MMTV promoter. Both strains of transgenic mice develop mammary tumors that appear with a shorter latency period when compared with animals expressing a MMTV/wild-type *neu* transgene (data not shown). These observations argue that mutations affecting conserved cysteine residues in this region of Neu can enhance transformation mediated by this receptor both *in vitro* and *in vivo*.

Like the cells expressing the Neu deletion mutants, stable cell lines expressing these point mutants of Neu exhibited elevated levels of tyrosine phosphorylated Neu which correlated with their ability to transform Rat-1 cells (ref. 22; data not shown). To explore whether the elevated levels of tyrosine phosphorylated Neu observed in these cells was due to enhanced receptor dimerization, Neu immunoprecipitates from cell lines expressing the three cysteine to serine mutations (Fig. 3*A*) were separated under both nonreducing and reducing conditions (Fig. 3 *B* and *C*). The results revealed that, like the Neu deletion mutants, mutation of cysteine 635 or 647 to serine resulted in the formation of Neu dimers under nonreducing conditions (Fig. 3*B*, lanes 4 and 6) which could be converted to the monomeric form by addition of reducing agents (Fig. 3*C*, lanes 4 and 6). In contrast, a majority of the Neu protein derived from cells expressing the weakly trans-

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Table 1. Transformation of Rat-1 cells by Neu receptors containing cysteine mutations

Expression plasmid	Focus Assay 1: Average no. of foci/μg*	Focus Assay 1: % transformation of pJ4ΩneuNT†	Focus Assay 2: Average no. of foci/μg*	Focus Assay 2: % transformation of pJ4ΩneuNT†	Focus Assay 3: Average no. of foci/μg*	Focus Assay 3: % transformation of pJ4ΩneuNT†	Relative transforming ability‡
pJ4ΩneuN	0	0	0	0	0	0	0
pJ4ΩneuNT	109 ± 5	100	219 ± 11§	100	144 ± 9	100	100 ± 0
pJ4Ωneu(C635S)	15 ± 3	14	38 ± 3	17	20 ± 2	14	15 ± 2
pJ4Ωneu(C639S)	3 ± 3	3	4 ± 1	2	2 ± 1	1	2 ± 1
pJ4Ωneu(C647S)	NA	NA	31 ± 2	14	14 ± 1	10	12 ± 3
pJ4ΩneuN	0	0	0	0	0	0	0
pJ4ΩneuNT	156 ± 5	100	160 ± 13	100	215 ± 19	100	100 ± 0
pJ4Ωneu(C635G)	21 ± 2	14	23 ± 1	15	31 ± 2	14	14 ± 1
pJ4Ωneu(C635M)	14 ± 2	.9	20 ± 4	12	37 ± 4	17	13 ± 4

Three independent focus assays were performed with Rat-1 fibroblasts. Each of the *neu* cDNAs were placed under the control of the Moloney murine leukemia virus long terminal repeat. The first two experiments were performed with the same large scale preparation of DNA while the third experiment was performed with DNA from an independent preparation. NA, data not available.
*Values listed represent the mean number of foci counted on six plates ± SD.
†Values represent the ratio of the mean number of foci obtained for each construct with respect to the activated form of *neu* (neuNT).
‡Values listed represent the mean transforming abilities from all three experiments ± SD.
§Values listed represent the mean number of foci counted on five plates ± SD.

forming C639S mutant Neu protein was found as a monomer under nonreducing conditions (Fig. 3*B*, lane 5). The receptors harboring these cysteine mutations display a reduced ability to dimerize when compared with the deletion mutant, 8340, which transforms at 22% of NeuNT (compare lanes 3 to 4–6). Taken together, these data argue that alteration of these conserved cysteine residues plays a crucial role in the oncogenic activation of Neu due to stimulation of disulfide bond mediated receptor dimerization.

The Disruption of Disulfide-Linked Dimers Results in Decreased Transformation of Rat-1 Cells by Altered Neu Receptors. Given that the presence of a reducing agent (2-mercaptoethanol) was capable of disrupting Neu dimers (Figs. 2*B* and 3*C*), we examined whether the addition of 2-mercaptoethanol could interfere with the ability of a Neu deletion mutant (8142) and a point mutant (C635S) to transform Rat-1 cells. Indeed, reducing agents have been used previously to interfere with signaling from activated versions of the throm-
F4 bopoietin receptor (27). As shown in Fig. 4, addition of increasing concentrations of 2-mercaptoethanol resulted in a dose-dependent decrease in the ability of both Neu mutants to transform cells. Addition of 500 μM of 2-mercaptoethanol virtually abolished the ability of both mutants to transform Rat-1 cells. To ensure that the decrease in the number of foci did not reflect a toxic effect of the reducing agent on the Rat-1 cells, we also tested the effect of 2-mercaptoethanol on the ability of polyomavirus middle T antigen to transform Rat-1 cells. Because polyomavirus middle T is primarily localized on the inner face of the cytoplasmic membrane (28), its transforming activity should not be affected by 2-mercaptoethanol. In contrast to the Neu mutants, addition of the same concentrations of 2-mercaptoethanol had no effect on the ability of polyomavirus middle T to induce focus formation (Fig. 4). These data demonstrate that disruption of receptor dimerization with reducing agents can dramatically interfere with the capacity of these Neu mutants to induce malignant transformation *in vivo*.

DISCUSSION

We have demonstrated that mutations affecting a highly conserved cysteine-rich region within the extracellular domain of Neu can activate its oncogenic potential. Our observations further suggest that the transforming activity of these mutant receptors is the result of the formation of disulfide-linked dimers. The ability of these mutants to transform cells correlates with their ability to induce receptor dimerization. Furthermore, we have shown that addition of a reducing agent to the culture medium can dramatically interfere with the ability of these mutant Neu receptors to induce transformed foci of Rat-1 cells. Taken together, these observations suggest that oncogenic activation of Neu in these mutant receptors occurs through a mechanism involving the constitutive formation of disulfide bond-mediated receptor dimers. It should be noted that the parental Rat-1 cells used in this study express both the EGFR and Neu (ref. 10; personal observations), albeit at low levels (10). Given that the mutant versions of Neu are expressed to much higher levels than the endogenous protein (Figs. 2*B* and 3*C*), we believe our data results from the formation of homodimers between the altered Neu receptors.

One potential explanation for our results is that alteration of the balance of cysteine residues within this region may disrupt the normal cysteine pairing that occurs in the unactivated receptor. Such a mutation would leave an unpaired cysteine residue that could participate in an intermolecular interaction with another altered receptor, resulting in a disulfide-linked dimer. Indeed, a similar mechanism has been proposed to account for the oncogenic activation of the Ret protooncogene in inherited forms of endocrine neoplasia type 2A (29). Although mutation of individual cysteine residues within this region of Neu resulted in its oncogenic activation, many of the Neu deletion mutations that removed these residues exhibited increased transforming activity. For example, five of the deletion mutants that remove or alter the cysteine at position 647 displayed elevated transforming activities compared with that of the C647S point mutation (22–56% compared with 12%). One potential explanation for this observation is that the spatial arrangement between the remaining cysteine residues also plays an important role in mediating receptor dimerization. For example, certain deletions may result in the free cysteine being exposed at the surface of the receptor where it is more accessible to the formation of an intermolecular disulfide bond.

The observation that deletion, insertion, or point mutation of cysteine residues within the juxtatransmembrane region of Neu results in receptor dimerization has important implications in understanding the mechanism of receptor tyrosine kinase activation. Significantly, this region displays homology among the remaining EGFR family members, particularly with respect to the position of the five conserved cysteine residues. Future experiments involving the generation of comparable mutations in the other EGFR family members will provide insights into whether these cysteine residues function in a similar manner. Given the capacity for Neu/ErbB-2 to form

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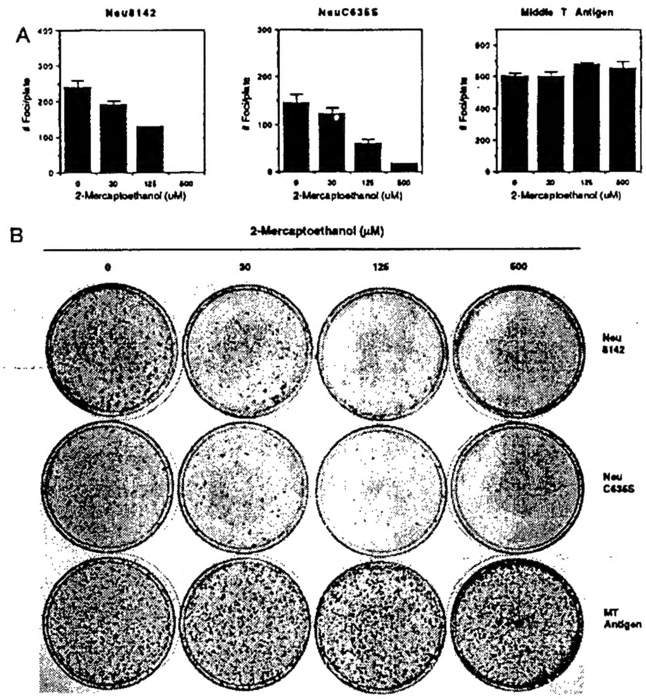

FIG. 4. Transformation mediated by mutant Neu receptors is impaired in the presence of reducing agents. (*A*) Focus assays were performed with a representative deletion (Neu8142) and point mutant (C635S) in the presence of increasing concentrations of 2-mercaptoethanol. Foci were scored over four plates for each concentration. The polyomavirus middle T antigen was used as a positive control. (*B*) Representative plates from the focus assay shown in *A* illustrating the decrease in transformed foci as a consequence of increasing 2-mercaptoethanol concentrations.

heterodimers (10–14), it is conceivable that these cysteine residues are also involved in heterodimerization between these closely related family members.

Further evidence implicating cysteine residues in the process of receptor dimerization stems from observations that the introduction of an extra cysteine residue proximal to the transmembrane domain of either the EGFR or Neu results in the formation of disulfide-linked dimers (30, 31). In contrast to our observations, the introduction of an extra cysteine residue in Neu did not activate the transforming ability of the altered receptor (31). The involvement of cysteine residues in receptor dimerization is further reinforced by the germline mutations within the *ret* protooncogene that occur in Men2A patients (32). These mutations affect one of five cysteine residues located in the extracellular domain of the Ret receptor. Subsequent analyses of these mutations revealed that they function by promoting receptor dimerization of the mutant receptors (29, 33). Finally, studies of receptors belonging to the haemopoietin/cytokine superfamily also indicate the importance of cysteine residues in receptor dimerization. The substitution of cysteine residues in place of specific residues in the extracellular domain of both the erythropoietin receptor (34, 35) and the thrombopoietin receptor, c-Mpl, (27) results in the formation of disulfide-linked dimers that constitutively activate these receptors. Furthermore, it has recently been demonstrated that disruption of disulfide bonding by the addition of reducing agents can interfere with signaling from activated thrombopoietin receptors (27). Consistent with this result, we have demonstrated that the addition of 2-mercaptoethanol can impair Neu-mediated transformation.

The observation that the transforming activity of these mutant Neu receptors can be severely impaired by the addition of reducing agents has potentially important therapeutic implications in the treatment of cancers overexpressing Neu. For example it may be possible to design peptide inhibitors that can effectively interfere with dimerization of the receptor by complexing with the critical cysteine residues within this region. Future studies directed toward elucidating the mechanism of disulfide-mediated receptor dimerization should provide important insights into the feasibility of this approach.

We thank Robert Weinberg for both the normal and activated *neu* cDNAs, Mark Greene for the 7.16.4 monoclonal antibody, Michael A. Rudnicki for the PGK-puro plasmid and Marc A. Webster for the pJ4ΩmT plasmid. We appreciate the technical support of Monica Graham, and we thank Brian Allore for automated DNA sequencing and Dinsdale Gooden for oligonucleotide synthesis (The Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University). We also thank John A. Hassell, Michael A. Rudnicki, and William R. Hardy for critical reading of the manuscript. This work was supported by a grant from the Breast Cancer Initiative (Sponsor Award No. 6287 401). W.J.M. is a recipient of a National Cancer Institute Scientist award and P.M.S holds a Medical Research Council scholarship.

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